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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US99/24826  <b>(22) International Filing Date:</b> 22 October 1999 (22.10.99)  <b>(30) Priority Data:</b> 60/105,507                      23 October 1998 (23.10.98)      US 60/108,685                      16 November 1998 (16.11.98)      US  <b>(71) Applicant (for all designated States except US):</b> MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02138 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> KAWASAKI, Hiroaki [JP/JP]; 3-20-2, Aoba, Higashi-ku, Fukuoka 813-0025 (JP). GRAYBIEL, Ann [US/US]; Boyce Farm Road, Lincoln, MA 01773 (US). HOUSMAN, David [US/US]; 64 Homer Street, Newton, MA 02158 (US).  <b>(74) Agent:</b> CAMACHO, Jennifer, A.; Testa, Hurwitz & Thibault, LLP, High Street Tower, 125 High Street, Boston, MA 02110 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> GENES INTEGRATING SIGNAL TRANSDUCTION PATHWAYS  <b>(57) Abstract</b>  <p>The present invention describes the identification, isolation, sequencing and characterization of two human CalDAG-GEF, and two human cAMP-GEF genes, which are associated with the Ras pathway. Also identified are CalDAG-GEF gene homologues in mice and cAMP-GEF gene homologues in rats. Nucleic acids and proteins comprising or derived from the CalDAG-GEFs and/or cAMP-GEFs are useful in screening and diagnosing certain Ras-associated cancers, in identifying and developing therapeutics for treatment of certain Ras-associated cancers, and in producing cell lines and transgenic animals useful as models of Ras-associated cancers.</p>		

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## GENES INTEGRATING SIGNAL TRANSDUCTION PATHWAYS

### Related Applications

This application claims the benefit of U.S. Application Nos. 60/105,507, filed on October 23, 1998, and 60/108,685, filed on November 16, 1998.

### Field of the Invention

The present invention relates generally to novel polynucleotides and proteins encoded by such polynucleotides, along with therapeutic, diagnostic, and research utilities for these polynucleotides and proteins.

### Background of the Invention

Ras proteins are key regulators of growth, differentiation and malignant transformation. In addition, these proteins are implicated in synaptic function and region-specific learning and memory functions in the brain.

As shown schematically in Figure 1, Ras proteins cycle between inactive GDP-complexed and active GTP-complexed states. GTPase-activating proteins (GAPs) inactivate Ras proteins by stimulating hydrolysis of the bound GTP to GDP, whereas guanine nucleotide exchange factors (GEFs) activate Ras proteins by stimulating release of GDP and the uptake of GTP. So essential are GEFs to Ras action, that genetic loss of GEF function has similar effects to those induced by loss of the Ras proteins themselves. Loss of GEF function can be circumvented by mutations that constitutively activate the Ras proteins, such as an oncogene mutation, or, in some cases, through loss of GAP activity. Activated Ras proteins, which are localized at the plasma membrane, transmit signals from tyrosine kinases to a cascade of serine/threonine kinases, which delivers the signals to the cell nucleus.

Activation of Ras can result in the activation of the mitogen-activated protein (MAP) kinase (also known as extracellular-signal regulated kinase, or ERK) pathway. For example, a receptor tyrosine kinase is activated by a peptide mitogen such as epidermal growth factor (EGF). The EGF-stimulated receptor undergoes autophosphorylation of specific tyrosine residues in its cytoplasmic domain which creates phosphotyrosyl binding sites for the Src homology 2 (SH2) and/or phosphotyrosyl binding (PTB) domains of certain adapter proteins. The adapter protein becomes autophosphorylated on association with activated receptor tyrosine kinases. The GEF is stably associated with the adapter protein which, upon autophosphorylation, mediates translocation of the GEF to the plasma membrane. The GEF then activates the Ras protein. Activated Ras relays its signal downstream through a cascade of cytoplasmic proteins, including

- 2 -

Raf-1 serine/threonine kinase. The Ras:Raf association promotes translocation of the normally cytoplasmic Raf protein to the plasma membrane, where subsequent events lead to the activation of its kinase function. Upon activation, Raf phosphorylates and activates two MAP kinases (also known as MEKs). MEKs directly associate with the catalytic domain of Raf-1 and are  
5 phosphorylated by Raf. Activated MEKs function as dual-specificity kinases and phosphorylate tandem threonine and tyrosine residues in the MAP kinases to activate them. Once activated, the MAP kinases translocate to the nucleus where they phosphorylate and activate a variety of substrates.

Rap proteins, members of the Ras small GTPase superfamily, can inhibit Ras signaling of  
10 the Ras/Raf-1(a serine/threonine kinase)/MAP kinase pathway or, through B-Raf, can activate MAP kinase. Rap1 consists of two isoforms, Rap1A and Rap1B, which differ mainly at the C-terminus. Characteristic features of Rap1 are its geranylgeranyl modification at the C-terminus, which is responsible for membrane attachments, and a threonine residue at position 61. In most other GTPases, the corresponding residue is a glutamine. Rap proteins, like Ras proteins, cycle  
15 between inactive GDP-complexed and active GTP-complexed states. GEFs are required to activate Rap proteins by stimulating the release of GDP and the uptake of GTP.

Constitutive activation of the Ras pathway contributes to malignant transformation. In fact, the Ras gene has been implicated in many human cancers, including lung cancer, breast cancer, colorectal cancer, exocrine pancreatic cancer, and myeloid leukemia. Biological and  
20 biochemical studies of Ras action indicate that Ras functions like a G-regulatory protein since Ras must be localized in the plasma membrane and must bind with GTP in order to transform cells. Gibbs et al., 53 MICROBIOL. REV. 171-286 (1989).

Targeting components of the Ras signaling pathways has been proposed as one approach for the development of anti-Ras drugs for cancer treatment. One potential approach for targeting  
25 Ras for cancer treatment involves the use of farnesyltransferase inhibitors (FTIs). Inhibition of farnesyl-protein transferase, and thereby of farnesylation of the Ras protein, blocks the ability of Ras to transform normal cells to cancer cells. Certain inhibitors of Ras farnesylation cause an increase in soluble Ras which can act as a dominant negative inhibitor of Ras function. While soluble Ras in cancer cells can become a dominant negative inhibitor, soluble Ras in normal cells  
30 would not be an inhibitor. A cytosol-localized and activated form of Ras acts as a dominant negative Ras inhibitor of membrane-bound Ras function. Gibbs et al., 86 PROC. NAT'L ACAD.

- 3 -

Sci. USA 6630-34 (1989). FTIs block Ras function by preventing its post-translational modification by the farnesyl isoprenoid.

Intervention of Ras signaling at multiple or various points can significantly impact the ability of Ras to cause cellular transformation. Since Ras protein function is believed to be crucial to so many cellular processes, targeting only a subset of Ras functions by downstream  
5 intervention may provide significant advantages. Thus, there remains a need for identifying additional means for disrupting the Ras pathway. Applicants have discovered four new targets, namely GEFs specific for Rap1A, for disrupting the Ras pathway.

#### Summary of the Invention

10 Applicants have discovered four mammalian genes which have been designated CalDAG-GEFI, CalDAG-GEFII, cAMP-GEFI, and cAMP-GEFII, which encode proteins having a substrate specificity for Rap1A. The proteins encoded by CalDAG-GEFI and CalDAG-GEFII, referred to herein generally as "CalDAG-GEF," have dual binding domains for calcium and diacylglycerol. The proteins cAMP-GEFI and cAMP-GEFII, referred to herein generally as  
15 "cAMP-GEF," have a binding domain for cyclic adenosine 3', 5'-monophosphate. The present disclosure provides polypeptide and polynucleotide sequences for Mus musculus CalDAG-GEFI, Homo sapiens CalDAG-GEFI, Rattus norvegicus CalDAG-GEFII, Homo sapiens CalDAG-GEFII, Rattus norvegicus cAMP-GEFI, Homo sapiens cAMP-GEFI, Homo sapiens alternatively spliced cAMP-GEFI, Rattus norvegicus cAMP-GEFII, and Homo sapiens cAMP-GEFII. See  
20 Kawasaki et al., 95 Proc. Natl. Acad. Sci. USA 13278-83 (1998), and Kawasaki et al., 282 Sci. 2275-79 (1998), the disclosures of both of which are incorporated by reference herein.

Thus, in one series of embodiments, the present invention provides isolated nucleic acids including nucleotide sequences comprising or derived from CalDAG-GEF or cAMP-GEF, or encoding polypeptides comprising or derived from CalDAG-GEF or cAMP-GEF proteins. The  
25 sequences of the invention include the specifically disclosed sequences, splice variants of these sequences, allelic variants of these sequences, synonymous sequences, and homologous or orthologous variants of these sequences. Thus, for example, the invention provides nucleic acid sequences from the Mus musculus CalDAG-GEFI, Homo sapiens CalDAG-GEFI, Rattus norvegicus CalDAG-GEFII, Homo sapiens CalDAG-GEFII, Rattus norvegicus cAMP-GEFI,  
30 Homo sapiens cAMP-GEFI, Homo sapiens alternatively spliced cAMP-GEFI, Rattus norvegicus cAMP-GEFII, and Homo sapiens cAMP-GEFII. The present invention also provides allelic variants and homologous or orthologous sequences by providing methods by which such variants

- 4 -

may be routinely obtained. Because the nucleic acids of the invention may be used in a variety of diagnostic, therapeutic and recombinant applications, various subsets of the CalDAG-GEF and cAMP-GEF sequences are also provided. For example, for use in allele specific hybridization screening or PCR amplification techniques, subsets of the CalDAG-GEF and cAMP-GEF sequences, including both sense and antisense sequences, and both normal and mutant sequences, as well as intronic, exonic and untranslated sequences, are provided. Such sequences may comprise a small number of consecutive nucleotides from the sequences which are disclosed or otherwise enabled herein, but preferably include at least 8-10, more preferably 10-15, and most preferably 15-25, consecutive nucleotides from a CalDAG-GEF or cAMP-GEF sequence. In another embodiment, such sequences include at least 25-500 consecutive nucleotides from CalDAG-GEF or cAMP-GEF sequence. Other preferred subsets of a CalDAG-GEF or cAMP-GEF sequence include those encoding one or more of the functional domains or antigenic determinants of the CalDAG-GEF or cAMP-GEF protein and, in particular, may include either normal (wild-type) or mutant sequences. The invention also provides for various nucleic acid constructs in which CalDAG-GEF or cAMP-GEF sequences, either complete or subsets, are operably joined to exogenous sequences to form cloning vectors, expression vectors, fusion vectors, transgenic constructs, and the like. Thus, in accordance with another aspect of the invention, a recombinant vector for transforming a mammalian or invertebrate tissue cell to express a normal or mutant CalDAG-GEF and/or cAMP-GEF sequence in the cells is provided.

In another series of embodiments, the present invention provides for host cells which have been transfected or otherwise transformed with one of the nucleic acids of the invention. The cells may be transformed merely for purposes of propagating the nucleic acid constructs of the invention, or may be transformed so as to express the CalDAG-GEF and/or cAMP-GEF sequences. The transformed cells of the invention may be used in assays to identify proteins and/or other compounds which affect normal or mutant CalDAG-GEF and/or cAMP-GEF expression, which interact with the normal or mutant CalDAG-GEF and/or cAMP-GEF proteins, and/or which modulate the function or effects of the normal or mutant proteins, or to produce the CalDAG-GEF and/or cAMP-GEF proteins, fusion proteins, functional domains, antigenic determinants, and/or antibodies of the invention. Transformed cells may also be implanted into hosts, including humans, for therapeutic or other reasons. Preferred host cells include mammalian cells, including pure or mixed cell cultures, as well as bacterial, yeast, nematode,



- 5 -

insect and other invertebrate cells. For uses as described below, preferred cells also include embryonic stem cells, zygotes, gametes, and germ line cells.

In another series of embodiments, the present invention provides transgenic animal models of diseases or disorders associated with mutations in the CalDAG-GEF and/or cAMP-GEF genes. The animal may be essentially any non-human mammal, including rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates. In addition, invertebrate models, including nematodes and insects, may be used for certain applications. The animal models are produced by standard transgenic methods including microinjection, electroporation, transfection, or other forms of transformation of embryonic stem cells, zygotes, gametes, and germ line cells with vectors including genomic or cDNA fragments, minigenes, homologous recombination vectors, viral insertion vectors and the like. Suitable vectors include vaccinia virus, adenovirus, adeno-associated virus, retrovirus, liposome transport, neuraltropic viruses, and Herpes simplex virus. The animal models may include transgenic sequences comprising or derived from the CalDAG-GEF and/or cAMP-GEF genes, including normal and mutant sequences, intronic, exonic and untranslated sequences, and sequences encoding subsets of the CalDAG-GEF and/or cAMP-GEF proteins, such as functional domains. The major types of animal models provided include: (1) Animals in which a normal human CalDAG-GEF and/or cAMP-GEF gene has been recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a recombinant gene or a large genomic fragment; in which a normal human CalDAG-GEF and/or cAMP-GEF gene has been recombinantly substituted for one or both copies of the animal's homologous CalDAG-GEF and/or cAMP-GEF gene by homologous recombination or gene targeting; and/or in which one or both copies of one of the animal's homologous CalDAG-GEF and/or cAMP-GEF genes have been recombinantly "humanized" by the partial substitution of sequences encoding the human homologue by homologous recombination or gene targeting; (2) Animals in which a mutant human CalDAG-GEF and/or cAMP-GEF gene has been recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a recombinant gene or a large genomic fragment; in which a mutant human CalDAG-GEF and/or cAMP-GEF gene has been recombinantly substituted for one or both copies of the animal's homologous CalDAG-GEF and/or cAMP-GEF gene by homologous recombination or gene targeting; and/or in which one or both copies of one of the animal's

- 6 -

homologous CalDAG-GEF and/or cAMP-GEF gene have been recombinantly "humanized" by the partial substitution of sequences encoding a mutant human homologue by homologous recombination or gene targeting; (3) Animals in which a mutant version of one of that animal's CalDAG-GEF or cAMP-GEF gene has been recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a recombinant gene or a large genomic fragment; and/or in which a mutant version of one of that animal's CalDAG-GEF or cAMP-GEF gene has been recombinantly substituted for one or both copies of the animal's homologous CalDAG-GEF or cAMP-GEF gene by homologous recombination or gene targeting; and (4) "Knock-out" animals in which one or both copies of one of the animal's CalDAG-GEF or cAMP-GEF genes have been partially or completely deleted by homologous recombination or gene targeting, or have been inactivated by the insertion or substitution by homologous recombination or gene targeting of exogenous sequences.

In another series of embodiments, the present invention provides for substantially pure protein preparations including polypeptides comprising or derived from the CalDAG-GEF and/or cAMP-GEF proteins. The CalDAG-GEF and cAMP-GEF protein sequences of the invention include the specifically disclosed sequences, variants of these sequences resulting from alternative mRNA splicing, allelic variants of these sequences, and homologous or orthologous variants of these sequences. Thus, for example, the invention provides amino acid sequences from the *Mus musculus* CalDAG-GEFI protein, *Homo sapiens* CalDAG-GEFI protein, *Rattus norvegicus* CalDAG-GEFII protein, *Homo sapiens* CalDAG-GEFII protein, *Rattus norvegicus* cAMP-GEFI protein, *Homo sapiens* cAMP-GEFI protein, *Homo sapiens* alternatively spliced cAMP-GEFI protein, *Rattus norvegicus* cAMP-GEFII protein, and *Homo sapiens* cAMP-GEFII protein. The present invention also provides allelic variants and homologous or orthologous proteins by providing methods by which such variants may be routinely obtained. The present invention also specifically provides for mutant or disease-causing variants of CalDAG-GEF and cAMP-GEF by providing methods by which such variants may be routinely obtained. Because the proteins of the invention may be used in a variety of diagnostic, therapeutic and recombinant applications, various subsets of the CalDAG-GEF and cAMP-GEF protein sequences and combinations of the CalDAG-GEF and cAMP-GEF protein sequences with heterologous sequences are also provided. For example, for use as immunogens or in binding assays, subsets of the CalDAG-GEF and cAMP-GEF protein sequences, including both normal and mutant

- 7 -

sequences, are provided. Such protein sequences may comprise a small number of consecutive amino acid residues from the sequences which are disclosed or otherwise enabled herein, but preferably include at least 4-8, and preferably at least 9-15 consecutive amino acid residues from a CalDAG-GEF or cAMP-GEF sequence. In another embodiment, such sequences comprise at least 15-200 consecutive amino acid residues from a CalDAG-GEF or cAMP-GEF sequence. Other preferred subsets of the CalDAG-GEF and cAMP-GEF protein sequences include those corresponding to one or more of the functional domains or antigenic determinants of the CalDAG-GEF and cAMP-GEF proteins and, in particular, may include either normal (wild-type) or mutant sequences. The invention also provides for various protein constructs in which a CalDAG-GEF and/or cAMP-GEF sequences, either complete or subsets thereof, are joined to exogenous sequences to form fusion proteins and the like. In accordance with these embodiments, the present invention also provides for methods of producing all of the above described proteins which comprise, or are derived from, CalDAG-GEF and/or cAMP-GEF.

In another series of embodiments, the present invention provides for the production and use of polyclonal and monoclonal antibodies, including antibody fragments, including Fab fragments, F(ab')<sub>2</sub>, and single chain antibody fragments, which selectively bind to CalDAG-GEF or cAMP-GEF, or to specific antigenic determinants of CalDAG-GEF or cAMP-GEF. The antibodies may be raised in mouse, rabbit, goat or other suitable animals, or may be produced recombinantly in cultured cells such as hybridoma cell lines. Preferably, the antibodies selectively bind to a sequence comprising at least 4-8, and preferably at least 9-15, consecutive amino acid residues from a CalDAG-GEF or cAMP-GEF sequence. The antibodies of the invention may be used in the various diagnostic, therapeutic and technical applications described herein.

In another series of embodiments, the present invention provides methods of screening or identifying proteins, small molecules or other compounds which are capable of inducing or inhibiting the expression and/or function of the CalDAG-GEF and/or cAMP-GEF genes or proteins. The assays may be performed *in vitro* using non-transformed cells, immortalized cell lines, or recombinant cell lines, or *in vivo* using the transgenic animal models enabled herein. In particular, the assays may detect the presence of increased or decreased expression of CalDAG-GEF and/or cAMP-GEF-related genes or proteins on the basis of increased or decreased mRNA expression, increased or decreased levels of CalDAG-GEF and/or cAMP-GEF-related protein products, or increased or decreased levels of expression of a marker gene (*e.g.*,  $\beta$ -galactosidase,

- 8 -

green fluorescent protein, alkaline phosphatase or luciferase) operably joined to a 5' regulatory region in a recombinant construct. Cells known to express CalDAG-GEF or cAMP-GEF, or transformed to express CalDAG-GEF or cAMP-GEF, are incubated and one or more test compounds are added to the medium. After allowing a sufficient period of time (*e.g.*, 0-72 hours) for the compound to induce or inhibit the expression of the CalDAG-GEF or cAMP-GEF, any change in levels of expression from an established baseline may be detected using any of the techniques described above. In particularly preferred embodiments, the cells are from an immortalized cell line such as a human neuroblastoma, glioblastoma or a hybridoma cell line, or are transformed cells of the invention.

In another series of embodiments, the present invention provides methods for identifying proteins and other compounds which bind to, or otherwise directly interact with, CalDAG-GEF and/or cAMP-GEF. The proteins and compounds will include endogenous cellular components which interact with the CalDAG-GEF and/or cAMP-GEF *in vivo* and which, therefore, provide new targets for pharmaceutical and therapeutic interventions, as well as recombinant, synthetic, and otherwise exogenous compounds which may have CalDAG-GEF and/or cAMP-GEF binding capacity and, therefore, may be candidates for pharmaceutical agents. Thus, in one series of embodiments, cell lysates or tissue homogenates (*e.g.*, human brain homogenates, lymphocyte lysates) may be screened for proteins or other compounds which bind to one of the normal or mutant CalDAG-GEF or cAMP-GEF proteins. Alternatively, any of a variety of exogenous compounds, both naturally occurring and/or synthetic (*e.g.*, libraries of small molecules or peptides), may be screened for CalDAG-GEF or cAMP-GEF binding capacity. In each of these embodiments, an assay is conducted to detect binding between a "CalDAG-GEF component" or a "cAMP-GEF component" and some other moiety. In one embodiment, a CalDAG-GEF component comprises a CalDAG-GEF SRC1, SRC2, SRC3, EF hand or a DAG-binding domain. In another embodiment, a cAMP-GEF component comprises a cAMP-GEF SRC1, SRC2, SRC3, or a cAMP-binding domain. The "CalDAG-GEF component" or the "cAMP-GEF component" in these assays may be any polypeptide comprising or derived from a normal or mutant CalDAG-GEF or cAMP-GEF protein, including functional domains or antigenic determinants of CalDAG-GEF or cAMP-GEF, or CalDAG-GEF or cAMP-GEF fusion proteins. Binding may be detected by non-specific measures (*e.g.*, changes in intracellular  $Ca^{2+}$ , GTP/GDP ratio) or by specific measures (*e.g.*, changes in the expression of downstream genes which can be monitored by differential display, 2D gel electrophoresis, differential hybridization, or SAGE methods). The

- 9 -

preferred methods involve variations on the following techniques: (1) direct extraction by affinity chromatography; (2) co-isolation of CalDAG-GEF or cAMP-GEF components and bound proteins or other compounds by immunoprecipitation; (3) the Biomolecular Interaction Assay (BIAcore); and (4) the yeast two-hybrid systems.

5 In another series of embodiments, the present invention provides for methods of identifying proteins, small molecules and other compounds capable of modulating the activity of normal or mutant CalDAG-GEF or cAMP-GEF. Using normal cells or animals, the transformed cells and transgenic animal models of the present invention, or cells obtained from subjects bearing normal or mutant CalDAG-GEF or cAMP-GEF genes, the present invention provides  
10 methods of identifying such compounds on the basis of their ability to affect the expression of CalDAG-GEF and/or cAMP-GEF, the intracellular localization of the CalDAG-GEF and/or cAMP-GEF, or other biochemical, histological, or physiological markers which distinguish cells bearing normal and mutant CalDAG-GEF and/or cAMP-GEF sequences. Using the transgenic animals of the invention, methods of identifying such compounds are also provided on the basis  
15 of the ability of the compounds to affect behavioral, physiological or histological phenotypes associated with mutations in CalDAG-GEF and/or cAMP-GEF.

In another series of embodiments, the present invention provides methods and reagents for the screening and diagnosis of diseases or disorders associated with mutations in the CalDAG-GEF and/or cAMP-GEF genes. Screening and/or diagnosis can be accomplished by  
20 methods based upon the nucleic acids (including genomic and mRNA/cDNA sequences), proteins, and/or antibodies disclosed and enabled herein, including functional assays designed to detect failure or augmentation of the normal CalDAG-GEF and/or cAMP-GEF activity and/or the presence of specific new activities conferred by the mutant CalDAG-GEF and/or cAMP-GEF. Thus, for example, screens and diagnostics based upon CalDAG-GEF and/or cAMP-GEF  
25 proteins are provided which detect differences between mutant and normal CalDAG-GEF or cAMP-GEF in electrophoretic mobility, in proteolytic cleavage patterns, in molar ratios of the various amino acid residues, or in ability to bind specific antibodies. In addition, screens and diagnostics based upon nucleic acids (gDNA, cDNA or mRNA) are provided which detect differences in nucleotide sequences by direct nucleotide sequencing, hybridization using allele  
30 specific oligonucleotides, restriction enzyme digest and mapping (*e.g.*, RFLP, REF-SSCP), electrophoretic mobility (*e.g.*, SSCP, DGGE), PCR mapping, RNase protection, chemical mismatch cleavage, ligase-mediated detection, and various other methods. Other methods are

- 10 -

also provided which detect abnormal processing of CalDAG-GEF and/or cAMP-GEF or proteins reacting with CalDAG-GEF and/or cAMP-GEF, alterations in CalDAG-GEF and/or cAMP-GEF transcription, translation, and post-translational modification; alterations in the intracellular and extracellular trafficking of CalDAG-GEF and/or cAMP-GEF gene products; or abnormal  
5 intracellular localization of CalDAG-GEF and/or cAMP-GEF. Such methods and reagents are also useful in the analysis of neoplasias and mammalian immune system function, as well as functional *in vivo* imaging of mammalian organ systems. In accordance with these embodiments, diagnostic kits are also provided which will include the reagents necessary for the above-described diagnostic screens.

10 In another series of embodiments, the present invention provides methods and therapeutic agents for use in the treatment of conditions such as neurological and neuropsychiatric disorders such as Huntington's disease, Parkinson's disease, Alzheimer's disease, dystonia, Tourette's syndrome, obsessive-compulsive disorder, attention deficit/hyperactive disorder, depression, schizophrenia, and stroke; neoplasias such as solid tumors including colon, breast, lung, prostate,  
15 and hematopoietic tumors such as leukemia, Hodgkins, and non-Hodgkins lymphomas; and autoimmune diseases, allergies, and asthma; as well as for the enhancement of the immune response in normal and immunocompromised individuals. These methods and therapeutic agents may be based upon (1) administration of normal CalDAG-GEF and/or cAMP-GEF proteins; (2) gene therapy with normal CalDAG-GEF and/or cAMP-GEF genes to compensate for or replace  
20 the mutant genes; (3) gene therapy based upon antisense sequences to mutant CalDAG-GEF and/or cAMP-GEF genes or upon sequences which "knock-out" the mutant genes; (4) gene therapy based upon sequences which encode a protein which blocks or corrects the deleterious effects of CalDAG-GEF and/or cAMP-GEF mutants; (5) immunotherapy based upon antibodies to normal and/or mutant CalDAG-GEF and/or cAMP-GEF proteins; or (6) small molecules  
25 (drugs) which alter CalDAG-GEF and/or cAMP-GEF expression, block interactions between (normal or mutant) forms of CalDAG-GEF and/or cAMP-GEF and other proteins or ligands, or which otherwise block the function of (normal or mutant) CalDAG-GEF and/or cAMP-GEF proteins by altering the structure of the proteins, by enhancing their metabolic clearance, or by inhibiting their function.

30 In accordance with another aspect of the invention, the proteins of the invention can be used as starting points for rational drug design to provide ligands, therapeutic drugs or other types of small chemical molecules. Alternatively, small molecules or other compounds

identified by the above-described screening assays may serve as "lead compounds" in rational drug design.

### Brief Description of the Drawings

Figure 1 is a partial schematic diagram of a Ras pathway.

5 Figure 2A shows human (h) and mouse (m) CalDAG-GEFI, human (h) and rat (r) CalDAG-GEFII, and *C. elegans* (cel) (F25B3.3, GenBank accession number: 1262950) CalDAG-GEF. Figure 2B shows a computer-generated phylogenetic tree analysis of the GEF domains of CalDAG-GEFI and CalDAG-GEFII in relation to other Ras-superfamily GEFs. Figure 2C shows multiple alignment of GEF structurally conserved regions (SCRs) of CalDAG-  
10 GEFs and several other GEFs of the Ras superfamily. Figure 2D shows the full-length amino acid sequences of human (h) and mouse (m) CalDAG-GEFI (box indicates amino acid differences). Figure 2E shows the sequence similarity (black indicates identity) of EF-hand domains in CalDAG-GEFs and other calcium binding proteins. Figure 2F shows the sequence similarity of DAG-binding domains of CalDAG-GEFs and PKC (protein kinase C) family  
15 proteins.

Figure 3A is a schematic representation of cAMP-GEF family proteins, including human (h) and rat (r) cAMP-GEFI, human (h) cAMP-GEFII and *C. elegans* (cel) (T2OG5.5, GenBank accession number: 458480) cAMP-GEF. Figure 3B is a phylogenetic tree analysis of cAMP binding domains of cAMP-GEFI and II and other cyclic nucleotide binding proteins. Figure 3C  
20 is a phylogenetic tree analysis of GEF domains of cAMP-GEFI and II and other Ras superfamily GEFs. Figure 3D shows the amino acid sequences of the structurally conserved regions (SCRs) of cAMP-GEFs and other Ras superfamily GEFs (black indicates identity). Figure 3E shows the amino acid sequences of the cAMP binding pockets of cAMP-GEFI and II and other cyclic nucleotide-binding proteins. The positions of invariant amino acid residues are shown by black  
25 diamonds. The open diamond indicates the amino acid that determines the binding specificity for cAMP or cGMP. The arrow indicates the position of amino acid substitutions specific to cAMP-GEFs. Figure 3F is the full-length amino acid sequences of human cAMP-GEFI and II (boxes indicate amino acid identity).

### Detailed Description of the Invention

30 The present invention is based, in part, upon the discovery of a family of mammalian genes which are associated with the Ras pathway. The discovery of these genes, designated

- 12 -

CalDAG-GEFI, CalDAG-GEFII, cAMP-GEFI, and cAMP-GEFII, as well as the characterization of these genes, their protein products, mutants, and possible functional roles, are described below.

### I. Definitions

5 In order to facilitate review of the various embodiments of the invention, and an understanding of the various elements and constituents used in making and using the invention, the following definitions are provided for particular terms used in the description and the claims which follow:

CalDAG-GEF. As used without further modification herein, the terms "CalDAG-GEF" or  
10 or "CalDAG-GEFs" refer to the CalDAG-GEFI and/or the CalDAG-GEFII genes/proteins. In particular, the unmodified terms "CalDAG-GEF" or "CalDAG-GEFs" refer to the mammalian genes/proteins and, preferably, the human genes/proteins.

cAMP-GEF. As used without further modification herein, the terms "cAMP-GEF" or  
15 "cAMP-GEFs" refer to the cAMP-GEFI and/or the cAMP-GEFII genes/proteins. In particular, the unmodified terms "cAMP-GEF" or "cAMP-GEFs" refer to the mammalian genes/proteins and, preferably, the human genes/proteins.

CalDAG-GEF gene. As used herein, the term "CalDAG-GEF gene" means the  
mammalian genes represented by SEQ ID NOS: 1, 3, 5, and 7, as well as any allelic variants and heterospecific mammalian homologues. A murine CalDAG-GEFI cDNA sequence is disclosed  
20 herein as SEQ ID NO: 1, and a human CalDAG-GEFI cDNA sequence is disclosed herein as SEQ ID NO: 3. A rat CalDAG-GEFII cDNA sequence is disclosed herein as SEQ ID NO: 5, and a human CalDAG-GEFII cDNA sequence is disclosed herein as SEQ ID NO: 7. The term "CalDAG-GEF gene" primarily relates to a coding sequence, but can also include some or all of the flanking regulatory regions and/or introns. The term "CalDAG-GEF gene" specifically  
25 includes artificial or recombinant genes created from cDNA or genomic DNA, including recombinant genes based upon splice variants.

CalDAG-GEF protein. As used herein, the term "CalDAG-GEF protein" means a protein encoded by a CalDAG-GEF gene, including allelic variants and heterospecific mammalian homologues. A murine CalDAG-GEFI protein sequence is disclosed herein as SEQ ID NO: 2,  
30 and a human CalDAG-GEFI protein sequence is disclosed herein as SEQ ID NO: 4. A rat CalDAG-GEFII protein sequence is disclosed herein as SEQ ID NO: 6, and a human CalDAG-GEFII protein sequence is disclosed herein as SEQ ID NO: 8. Splice variants are also embraced



- 13 -

by the term CalDAG-GEF protein as used herein. The protein may be produced by recombinant cells or organisms, may be substantially purified from natural tissues or cell lines, or may be synthesized chemically or enzymatically. Therefore, the term "CalDAG-GEF protein" is intended to include the protein in glycosylated, partially glycosylated, or unglycosylated forms, as well as in phosphorylated, partially phosphorylated, unphosphorylated, sulphated, partially sulphated, or unsulphated forms. The term also includes allelic variants and other functional equivalents of the CalDAG-GEF amino acid sequences, including biologically active proteolytic or other fragments.

hCalDAG-GEF gene and/or protein. As used herein, the abbreviation "hCalDAG-GEF" refers to the human homologue and human allelic variants of the CalDAG-GEF genes and/or proteins. Two cDNA sequences of the human CalDAG-GEF genes are disclosed herein as SEQ ID NOS: 3 and 7. The corresponding hCalDAG-GEF protein sequences are disclosed herein as SEQ ID NOS: 4 and 8. Allelic variants, including deleterious mutants, are enabled in the description which follows.

mCalDAG-GEF gene and/or protein. As used herein, the abbreviation "mCalDAG-GEF" refers to the murine homologues and murine allelic variants of the CalDAG-GEF gene and/or protein. A cDNA sequence of one murine CalDAG-GEF gene is disclosed herein as SEQ ID NO: 16. The corresponding mCalDAG-GEF protein sequence is disclosed herein as SEQ ID NO: 17. Allelic variants, including deleterious mutants, are enabled in the description which follows.

rCalDAG-GEF gene and/or protein. As used herein, the abbreviation "rCalDAG-GEF" refers to the rat homologue and rat allelic variants of the CalDAG-GEF genes and/or proteins. A cDNA sequence of one rat CalDAG-GEF gene is disclosed herein as SEQ ID NO: 5. The corresponding rCalDAG-GEF protein sequence is disclosed herein as SEQ ID NO: 6. Allelic variants, including deleterious mutants, are enabled in the description which follows.

cAMP-GEF gene. As used herein, the term "cAMP-GEF gene" means the mammalian genes represented by SEQ ID NOS: 9, 11, 13, 15, and 17, as well as any allelic variants and heterospecific mammalian homologues. A rat cAMP-GEFI cDNA sequence is disclosed herein as SEQ ID NO: 9, and a human cAMP-GEFI cDNA sequence is disclosed as SEQ ID NO: 11.

Another human cAMP-GEFI cDNA sequence, resulting from alternative splicing of the mRNA transcript, is disclosed as SEQ ID NO: 13. A rat cAMP-GEFII cDNA sequence is disclosed as SEQ ID NO: 15, and a human cAMP-GEFII cDNA sequence is disclosed as SEQ ID NO: 17.

- 14 -

The term "cAMP-GEF gene" primarily relates to a coding sequence, but can also include some or all of the flanking regulatory regions and/or introns. The term cAMP-GEF gene specifically includes artificial or recombinant genes created from cDNA or genomic DNA, including recombinant genes based upon splice variants.

5        cAMP-GEF protein. As used herein, the term "cAMP-GEF protein" means a protein encoded by a cAMP-GEF gene, including allelic variants and heterospecific mammalian homologues. A rat cAMP-GEFI protein sequence is disclosed herein as SEQ ID NO: 10, and a human cAMP-GEFI protein sequence is disclosed as SEQ ID NO: 12. Another human cAMP-GEFI protein sequence, resulting from alternative splicing of the mRNA transcript, is disclosed  
10 as SEQ ID NO: 14. A rat cAMP-GEFII protein sequence is disclosed as SEQ ID NO: 16, and a human cAMP-GEFII protein sequence is disclosed as SEQ ID NO: 18. Splice variants are also embraced by the term cAMP-GEF protein as used herein. The protein may be produced by recombinant cells or organisms, may be substantially purified from natural tissues or cell lines, or may be synthesized chemically or enzymatically. Therefore, the term "cAMP-GEF protein" is  
15 intended to include the protein in glycosylated, partially glycosylated, or unglycosylated forms, as well as in phosphorylated, partially phosphorylated, unphosphorylated, sulphated, partially sulphated, or unsulphated forms. The term also includes allelic variants and other functional equivalents of the cAMP-GEF amino acid sequences, including biologically active proteolytic or other fragments.

20        hcAMP-GEF gene and/or protein. As used herein, the abbreviation "hcAMP-GEF" refers to the human homologue and human allelic variants of the cAMP-GEF gene and/or protein. One cDNA sequences of the human cAMP-GEF gene is disclosed herein as SEQ ID NO: 18. The corresponding hcAMP-GEF protein sequence is disclosed herein as SEQ ID NO: 19. Numerous allelic variants, including deleterious mutants, are disclosed and enabled throughout the  
25 description which follows.

rcAMP-GEF gene and/or protein. As used herein, the abbreviation "rcAMP-GEF" refers to the rat homologue and rat allelic variants of the cAMP-GEF gene and/or protein. Two cDNA sequences of rat cAMP-GEF genes are disclosed herein as SEQ ID NOS: 9 and 15. The corresponding rcAMP-GEF protein sequences are disclosed herein as SEQ ID NOS: 10 and 16..  
30 Numerous allelic variants, including deleterious mutants, are disclosed and enabled throughout the description which follows.

- 15 -

Normal. As used herein with respect to genes, the term "normal" refers to a gene which encodes and expresses a normal protein. As used herein with respect to proteins, the term "normal" means a protein which performs its usual or normal physiological role and which is not associated with, or causative of, a pathogenic condition or state. Therefore, as used herein, the term "normal" is essentially synonymous with the usual meaning of the phrase "wild type." For any given gene, or corresponding protein, a multiplicity of normal allelic variants may exist, none of which is associated with the development of a pathogenic condition or state. Such normal allelic variants include, but are not limited to, variants in which one or more nucleotide substitutions do not result in a change in the encoded amino acid sequence.

Mutant. As used herein with respect to genes, the term "mutant" refers to a gene which encodes a mutant protein and/or fails to express a normal protein. As used herein with respect to proteins, the term "mutant" means a protein which does not perform its usual or normal physiological role and which is associated with, or causative of, a pathogenic condition or state. Therefore, as used herein, the term "mutant" is essentially synonymous with the terms "dysfunctional," "pathogenic," "disease-causing," and "deleterious." With respect to the CalDAG-GEF and cAMP-GEF genes and proteins of the present invention, the term "mutant" refers to CalDAG-GEF and cAMP-GEF genes/proteins bearing one or more nucleotide/amino acid substitutions, insertions and/or deletions which cause the genes/proteins to be dysfunctional, pathogenic, disease-causing or otherwise deleterious. This definition is understood to include the various mutations that naturally exist, including but not limited to those disclosed herein, as well as synthetic or recombinant mutations produced by human intervention. The term "mutant," as applied to the CalDAG-GEF and cAMP-GEF genes, is not intended to embrace sequence variants which, due to the degeneracy of the genetic code, encode proteins identical to the normal sequences disclosed or otherwise enabled herein; nor is it intended to embrace sequence variants which, although they encode different proteins, encode proteins which are functionally equivalent to normal CalDAG-GEF and/or cAMP-GEF proteins.

Functional equivalent. As used herein in describing gene sequences and amino acid sequences, the term "functional equivalent" means that a recited sequence need not be identical to a particularly disclosed sequence of the SEQ ID NOs but need only provide a sequence which functions biologically and/or chemically as the equivalent of the disclosed sequence.

Substantially pure. As used herein with respect to protein preparations, the term "substantially pure" means a preparation which contains at least 60% (by dry weight) the protein

- 16 -

of interest, exclusive of the weight of other intentionally included compounds. Preferably the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by dry weight the protein of interest, exclusive of the weight of other intentionally included compounds. Purity can be measured by any appropriate method, e.g., column chromatography, gel electrophoresis, or HPLC analysis. If a preparation intentionally includes two or more different proteins of the invention, a "substantially pure" preparation means a preparation in which the total dry weight of the proteins of the invention is at least 60% of the total dry weight, exclusive of the weight of other intentionally included compounds. Preferably, for such preparations containing two or more proteins of the invention, the total weight of the proteins of the invention be at least 75%, more preferably at least 90%, and most preferably at least 99%, of the total dry weight of the preparation, exclusive of the weight of other intentionally included compounds. Thus, if the proteins of the invention are mixed with one or more other proteins (e.g., serum albumin, 6-OST) or compounds (e.g., diluents, detergents, excipients, salts, polysaccharides, sugars, lipids) for purposes of administration, stability, storage, and the like, the weight of such other proteins or compounds is ignored in the calculation of the purity of the preparation.

Isolated nucleic acid. As used herein, an "isolated nucleic acid" is a ribonucleic acid, deoxyribonucleic acid, or nucleic acid analog comprising a polynucleotide sequence that has been isolated or separated from sequences that are immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant nucleic acid which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequences and/or including exogenous regulatory elements.

Transformed cell. As used herein, a "transformed cell" is a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a nucleic acid molecule of interest. The nucleic acid of interest will typically encode a peptide or protein. The transformed cell may express the sequence of interest or may be used only to propagate the sequence. The term "transformed" may be used herein to embrace any method of introducing

- 17 -

exogenous nucleic acids including, but not limited to, transformation, transfection, electroporation, microinjection, viral-mediated transfection, and the like.

Operably joined. As used herein, a coding sequence and a regulatory region are said to be "operably joined" when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory region. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of promoter function results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the regulatory region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a regulatory region would be operably joined to a coding sequence if the regulatory region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

Stringent hybridization conditions. Stringent hybridization conditions is a term of art understood by those of ordinary skill in the art. For any given nucleic acid sequence, stringent hybridization conditions are those conditions of temperature, chaotropic acids, buffer, and ionic strength which will permit hybridization of that nucleic acid sequence to its complementary sequence and not to substantially different sequences. The exact conditions which constitute "stringent" conditions, depend upon the nature of the nucleic acid sequence, the length of the sequence, and the frequency of occurrence of subsets of that sequence within other non-identical sequences. By varying hybridization conditions from a level of stringency at which non-specific hybridization occurs to a level at which only specific hybridization is observed, one of ordinary skill in the art can, without undue experimentation, determine conditions which will allow a given sequence to hybridize only with complementary sequences. Suitable ranges of such stringency conditions are described in KRAUSE ET AL., METHODS IN ENZYMOLOGY, 200: 546-56 (1991). Stringent hybridization conditions, depending upon the length and commonality of a sequence, may include temperatures of 20°C-65°C and ionic strengths from 5x to 0.1x SSC. Highly stringent hybridization conditions may include temperatures as low as 40-42°C (when denaturants such as formamide are included) or up to 60-65°C in ionic strengths as low as 0.1x SSC. These ranges, however, are only illustrative and, depending upon the nature of the target sequence, and possible future technological developments, may be more stringent than necessary.

- 18 -

Less than stringent conditions are employed to isolate nucleic acid sequences which are substantially similar, allelic or homologous to any given sequence.

Selectively bind. As used herein with respect to antibodies, an antibody is said to "selectively bind" to a target if the antibody recognizes and binds the target of interest but does not substantially recognize and bind other molecules in a sample, *e.g.*, a biological sample, which includes the target of interest.

CalDAG-GEF- or cAMP-GEF-associated disorder, condition, or disease. As used herein, the term "CalDAG-GEF or cAMP-GEF associated disorder, condition, or disease" means any disorder, condition, or disease to which a normal or mutant CalDAG-GEF and/or cAMP-GEF is related in any manner, such as in the causation, prevention, exacerbation, alleviation of the disorder. Thus, as used herein, a CalDAG-GEF- or cAMP-GEF-associated disorder, condition, or disease includes disorders related to the Ras-pathway, such as Ras-related cancers.

Adapter protein. As used herein, the term "adapter protein" means any protein that binds or is bound to a CalDAG-GEF or a cAMP-GEF protein, and facilitates localization of the bound CalDAG-GEF or cAMP-GEF at the plasma membrane, thereby facilitating Ras activation.

Variant. As used herein a "variant" sequence has, or will result in having, a sufficient amino acid similarity to have a reasonable expectation of success in the methods of the present invention. In order to produce variants of the disclosed sequences that may also functionally serve as a CalDAG-GEF or cAMP-GEF protein, any one or more of the naturally-occurring CalDAG-GEF or cAMP-GEF sequences disclosed herein may be used as a reference sequence to determine whether a candidate sequence possesses sufficient amino acid similarity to have a reasonable expectation of success in the methods of the present invention. Preferably, variant sequences are at least 70% similar or 60% identical, more preferably at least 75% similar or 65% identical, and most preferably 80% similar or 70% identical to one of the disclosed, naturally-occurring sequences.

To determine whether a candidate peptide region has the requisite percentage similarity or identity to a reference polypeptide or peptide oligomer, the candidate amino acid sequence and the reference amino acid sequence are first aligned using the dynamic programming algorithm described in Smith and Waterman (1981), *J. Mol. Biol.* 147:195-197, in combination with the BLOSUM62 substitution matrix described in Figure 2 of Henikoff and Henikoff (1992), "Amino acid substitution matrices from protein blocks", *PNAS* (1992 Nov), 89:10915-10919. For the present invention, an appropriate value for the gap insertion penalty is -12, and an appropriate

- 19 -

value for the gap extension penalty is -4. Computer programs performing alignments using the algorithm of Smith-Waterman and the BLOSUM62 matrix, such as the GCG program suite (Oxford Molecular Group, Oxford, England), are commercially available and widely used by those skilled in the art.

5           Once the alignment between the candidate and reference sequence is made, a percent similarity score may be calculated. The individual amino acids of each sequence are compared sequentially according to their similarity to each other. If the value in the BLOSUM62 matrix corresponding to the two aligned amino acids is zero or a negative number, the pairwise similarity score is zero; otherwise the pairwise similarity score is 1.0. The raw similarity score is  
10 the sum of the pairwise similarity scores of the aligned amino acids. The raw score is then normalized by dividing it by the number of amino acids in the smaller of the candidate or reference sequences. The normalized raw score is the percent similarity. Alternatively, to calculate a percent identity, the aligned amino acids of each sequence are again compared sequentially. If the amino acids are non-identical, the pairwise identity score is zero; otherwise  
15 the pairwise identity score is 1.0. The raw identity score is the sum of the identical aligned amino acids. The raw score is then normalized by dividing it by the number of amino acids in the smaller of the candidate or reference sequences. The normalized raw score is the percent identity. Insertions and deletions are ignored for the purposes of calculating percent similarity and identity. Accordingly, gap penalties are not used in this calculation, although they are used  
20 in the initial alignment.

In all instances, variants of the naturally-occurring CalDAG-GEF or cAMP-GEF proteins, as described above, must be tested for biological activity as described below. Specifically, the proteins must exhibit guanine nucleotide exchange factor activity, and, preferably, they have the ability to inhibit Ras signaling of the Ras/Raf-1/MAP kinase pathway.

## 25   II. The CalDAG-GEFs

CalDAG-GEFI has a substrate specificity for Rap1A, dual binding domains for calcium ( $\text{Ca}^{2+}$ ) and diacylglycerol (DAG), and enriched expression in brain basal ganglia pathways and their axon-terminal regions. Expression of CalDAG-GEFI activates Rap1A and inhibits Ras-dependent activation of the Erk/MAP kinase cascade in 293T cells.  $\text{Ca}^{2+}$  ionophore and phorbol  
30 ester strongly and additively enhance this Rap1A activation. By contrast, CalDAG-GEFII exhibits a different brain expression pattern and fails to activate Rap1A, but activates H-Ras, R-Ras and the Erk/MAP kinase cascade under  $\text{Ca}^{2+}$  and DAG modulation. The CalDAG-GEF

proteins have a critical neuronal function in determining the relative activation of Ras and Rap1 signaling induced by  $\text{Ca}^{2+}$  and DAG mobilization. The expression of CalDAG-GEFI and CalDAG-GEFII in hematopoietic organs indicates that such control has broad significance in Ras/Rap regulation of normal and malignant states.

5       The basal ganglia are centrally implicated in movement control and in forms of procedural learning related to habit formation. It is not yet known whether particular neurochemical specializations of the basal ganglia contribute to these behavioral functions. The basal ganglia do, however, have a unique double-inhibitory pathway design combined with abundant expression of neuromodulators in striatal neurons. A number of genes with  
10   differentially high expression in the striatum have also been identified. These include genes coding for proteins with signaling functions, such as adenylate cyclase V (Glatt et al., 361 NATURE (LONDON), 536-38 (1993)) and DARPP-32 (Hemmings et al., 310 NATURE (LONDON) 502-05 (1984)). To identify other cellular signaling molecules that could contribute to basal ganglia functions, a search for striatum-enriched transcripts was performed by a differential  
15   display method, as discussed in Example 1. Among the transcripts identified in this search were a family of genes characterized by the presence of a Ras superfamily (GEF) domain.

Specific domains identified include structurally conserved GEF regions SCR1, SCR2, and SCR3, as shown in Figures 2C and 3D, and as shown in the following table.



- 21 -

TABLE 1

Gene	SCR1	SCR2	SCR3
hCalDAG-GEFI	SEQ ID NO.3: 605-677 SEQ ID NO.4: 149-173	SEQ ID NO.3: 817-946 SEQ ID NO.4: 219-262	SEQ ID NO.3: 1053-1185 SEQ ID NO.4: 298-320
hCalDAG-GEFII	SEQ ID NO.7: 728-800 SEQ ID NO.8: 205-229	SEQ ID NO.7: 913-1042 SEQ ID NO.8: 270-313	SEQ ID NO.7: 1084-1216 SEQ ID NO.8: 348-371
hcAMP-GEFI	SEQ ID NO.11: 2058-2130 SEQ ID NO.12: 205-229	SEQ ID NO.11: 2276-2405 SEQ ID NO.12: 688-731	SEQ ID NO.11: 2516-2582 SEQ ID NO.12: 767-789
rcAMP-GEFI	SEQ ID NO.9: 2050-2122 SEQ ID NO.10: 618-642	SEQ ID NO.9: 2267-2396 SEQ ID NO.10: 691-734	SEQ ID NO.9: 2502-2568 SEQ ID NO.10: 770-792
hcAMP-GEFII	SEQ ID NO.17: 2707-2779 SEQ ID NO.18: 767-791		
rcAMP-GEFII	SEQ ID NO.15: 576-648 SEQ ID NO.16: 192-216		

In addition, the EF hand and DAG-binding domains were identified as shown in Figures 2E and 2F, and as shown in the following table:

- 22 -

Table 2

Gene	EF Hand Domain	DAG-Binding Domain
hCalDaG-GEFI	SEQ ID NO.3: 1456-1516 SEQ ID NO.4: 432-452	SEQ ID NO.3: 1652-1804 SEQ ID NO.4: 498-548
hCalDAG-GEFII	SEQ ID NO.7: 1384-1444 SEQ ID NO.8: 427-447	SEQ ID NO.7: 1579-1729 SEQ ID NO.8: 492-542

Finally, the cAMP-binding domains were identified as shown in Figure 3E, and as shown in the following table:

Table 3

Gene	cAMP-Binding Domain
hcAMP-GEFI	SEQ ID NO.11: 2012-2255 SEQ ID NO.12: 219-300
rcAMP-GEFI	SEQ ID NO.9: 853-1096 SEQ ID NO.10: 219-300
rcAMP-GEFII	SEQ ID NO.17: 1522-1765 SEQ ID NO.18: 372-453

### III. The cAMP-GEFs

Cyclic adenosine 3', 5'-monophosphate (cAMP) is a universal second messenger that induces a variety of physiological responses in eukaryotic cells ranging from growth, differentiation, and gene expression to secretion and neurotransmission. The cAMP second messenger system has also been centrally implicated in modulating synaptic function, neuroplasticity and learning and memory. Most of these effects have been attributed to the binding of cAMP to cAMP-dependent protein kinase (PKA), leading in turn to the activation of

- 23 -

intracellular phosphorylation cascades. Reported herein is the identification of a new family of cAMP binding proteins that are differentially distributed in the brain and body organs and that are characterized by the presence of both a cAMP binding domain and a guanine nucleotide exchange factor (GEF) domain. These proteins, cAMP-GEFs, bind cAMP and selectively  
5 activate the Ras superfamily small G protein, Rap1A, in a cAMP-dependent but PKA-independent manner.

The general concept of cAMP signaling involves the sequential activation (or inhibition) of cAMP production by G proteins, the binding of cAMP to PKA, and the triggering of a series of downstream serine-threonine phosphorylation cascades. Viewed as the nearly exclusive  
10 target of cAMP binding in eukaryotic cells, PKA has been considered the essential effector molecule mediating a wide range of physiological effects of G protein/cAMP-triggered phosphorylation cascades. As the main cAMP effector, PKA has also been shown to function in the indirect coupling of the cAMP signal transduction system to other intracellular signaling cascades. The cAMP signaling system has also been strongly implicated in neuronal functions  
15 ranging from neurotransmitter-initiated signaling to neuroplasticity underlying development and memory, but PKA has not been clearly linked to all of these neuronal functions, and region-specific neuronal effects have been observed as well. The cAMP-GEF gene has a Ras superfamily GEF motif. Thus, the gene codes for a novel cAMP binding protein that directly couples the cAMP signal transduction system to Ras superfamily cascades.

#### 20 IV. Preferred Embodiments

Based, in part, upon the discoveries disclosed and described herein, the following preferred embodiments of the present invention are provided.

##### 1. Isolated Nucleic Acids

In one series of embodiments, the present invention provides isolated nucleic acids  
25 corresponding to, or relating to, the CalDAG-GEF or cAMP-GEF nucleic acid sequences disclosed herein. As described more fully below, these sequences include normal CalDAG-GEF and cAMP-GEF sequences from humans and other mammalian species, mutant CalDAG-GEF and cAMP-GEF sequences from humans and other mammalian species, homologous sequences from non-mammalian species such as *Drosophila* and *C. elegans*, subsets of these sequences  
30 useful as probes and PCR primers, subsets of these sequences encoding fragments of the CalDAG-GEF or cAMP-GEF proteins or corresponding to particular domains or polymorphic

- 24 -

regions, complementary or antisense sequences corresponding to fragments of the CalDAG-GEF or cAMP-GEF genes, sequences in which the CalDAG-GEF and/or cAMP-GEF coding regions have been operably joined to exogenous regulatory regions, and sequences encoding fusion proteins of the portions of the CalDAG-GEF or cAMP-GEF proteins fused to other proteins useful as markers of expression, as "tags" for purification, or in screens and assays for proteins interacting with the CalDAG-GEFs and/or cAMP-GEFs.

Thus, in a first series of embodiments, isolated nucleic acid sequences are provided which encode normal versions of the CalDAG-GEF and cAMP-GEF proteins. Examples of such nucleic acid sequences are disclosed herein. These nucleic acids may be genomic sequences or may be cDNA sequences (*e.g.*, SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17). Thus, for example, the invention provides nucleic acid sequences in which the alternative splice variants described herein are incorporated at the DNA level, thereby, enabling cells including these sequences to express only one of the alternative splice variants at each splice position. For example, a recombinant gene may be produced in which one of the splice variants of cAMP-GEF is incorporated into DNA such that cells having this recombinant gene can express only one of these variants. For purposes of reducing the size of a recombinant CalDAG-GEF or cAMP-GEF gene, a cDNA gene may be employed or various combinations of the introns and untranslated exons may be removed from a DNA construct. Such constructs may be particularly useful, as described below, in identifying compounds which can induce or repress the expression of the CalDAG-GEF or cAMP-GEF genes.

In addition to the disclosed CalDAG-GEF and/or cAMP-GEF sequences, one of ordinary skill in the art is now enabled to identify and isolate nucleic acids corresponding to CalDAG-GEF or cAMP-GEF genes or cDNAs which are allelic to the disclosed sequences or which are heterospecific homologues. Thus, the present invention provides isolated nucleic acids corresponding to these alleles and homologues, as well as various recombinant constructs derived from these sequences, by means which are well known in the art. Briefly, one of ordinary skill in the art may now screen preparations of genomic or cDNA, including samples prepared from individual organisms (*e.g.*, human cancer patients or their family members) as well as bacterial, viral, yeast or other libraries of genomic or cDNA, using probes or PCR primers to identify allelic or homologous sequences. Because it is desirable to identify additional CalDAG-GEF and/or cAMP-GEF gene mutations which may contribute to the development of Ras-related cancers, because it is desirable to identify additional CalDAG-GEF and/or cAMP-

- 25 -

GEF polymorphisms which are not mutant or have antitumorigenic effects, and because it is also desired to create a variety of animal models which may be used to study Ras-related cancers and screen for potential therapeutics, it is particularly contemplated that additional CalDAG-GEF and/or cAMP-GEF sequences will be isolated from other preparations or libraries of human  
5 nucleic acids and from preparations or libraries from animals including rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates. Furthermore, CalDAG-GEF and/or cAMP-GEF homologues from yeast or invertebrate species, including *C. elegans* and other nematodes, as well as *Drosophila* and other insects, may have particular utility for drug screening. For example, invertebrates bearing mutant CalDAG-GEF and/or cAMP-GEF  
10 homologues (or mammalian CalDAG-GEF and/or cAMP-GEF transgenes) which cause a rapidly occurring and easily scored phenotype (*e.g.*, abnormal eye development after several days) can be used as screens for drugs which block the effect of the mutant gene. Such invertebrates may prove far more rapid and efficient for mass screenings than larger vertebrate animals. Once lead compounds are found through such screens, they may be tested in higher animals.

15 Depending upon the intended use, the present invention provides nucleic acid subsequences of the CalDAG-GEF and/or cAMP-GEF genes which may have lengths varying from 8-10 nucleotides (*e.g.*, for use as PCR primers) to nearly the full size of the CalDAG-GEF and/or cAMP-GEF genes. Thus, the present invention provides isolated nucleic acids comprising sequences corresponding to at least 8, preferably at least 10, and more preferably at least 15  
20 consecutive nucleotides of the CalDAG-GEF and/or cAMP-GEF genes, as disclosed or otherwise enabled herein, or to their complements.

In another series of embodiments, the present invention provides for isolated nucleic acids encoding all or a portion of the CalDAG-GEF and/or cAMP-GEF proteins in the form of a fusion protein. In these embodiments, a nucleic acid regulatory region (endogenous or  
25 exogenous) is operably joined to a first coding region which is covalently joined in-frame to a second coding region. The CalDAG-GEF and/or cAMP-GEF sequences of the fusion protein may represent the first, second, or any additional coding regions. The CalDAG-GEF and/or cAMP-GEF sequences may be conserved or non-conserved domains and can be placed in any coding region for the fusion protein.

30 In another series of embodiments, the present invention provides isolated nucleic acids in the form of recombinant DNA constructs in which a marker or reporter gene (*e.g.*,  $\beta$ -galactosidase, luciferase) is operably joined to the 5' regulatory region of a CalDAG-GEF and/or

- 26 -

cAMP-GEF gene such that expression of the marker gene is under the control of the CalDAG-GEF and/or cAMP-GEF regulatory sequences. Such isolated nucleic acids may be used to produce cells, cell lines or transgenic animals which are useful in the identification of compounds which can, directly or indirectly, differentially affect the expression of the CalDAG-GEFs and/or cAMP-GEFs.

Finally, the isolated nucleic acids of the present invention include any of the above described sequences when included in vectors. Appropriate vectors include cloning vectors and expression vectors of all types, including plasmids, phagemids, cosmids, episomes, and the like, as well as integration vectors. The vectors may also include various marker genes (*e.g.*, antibiotic resistance or susceptibility genes) which are useful in identifying cells successfully transformed therewith. In addition, the vectors may include regulatory sequences to which the nucleic acids of the invention are operably joined, and/or may also include coding regions such that the nucleic acids of the invention, when appropriately ligated into the vector, are expressed as fusion proteins. Such vectors may also include vectors for use in yeast "two hybrid," baculovirus, and phage-display systems.

## 2. Substantially Pure Proteins

The present invention provides for substantially pure preparations of the CalDAG-GEF and/or cAMP-GEF proteins, fragments of the CalDAG-GEF and/or cAMP-GEF proteins, and fusion proteins including the CalDAG-GEFs and/or cAMP-GEFs or fragments thereof. The proteins, fragments and fusions have utility, as described herein, in the generation of antibodies to normal and mutant CalDAG-GEFs and/or cAMP-GEFs, in the identification of CalDAG-GEF and/or cAMP-GEF binding proteins, and in diagnostic and therapeutic methods. Therefore, depending upon the intended use, the present invention provides substantially pure proteins or peptides comprising amino acid sequences which are subsequences of the complete CalDAG-GEF and/or cAMP-GEF proteins and which may have lengths varying from 4-8 amino acids (*e.g.*, for use as immunogens), or 9-15 amino acids (*e.g.*, for use in binding assays), to the complete CalDAG-GEF and/or cAMP-GEF proteins. Thus, the present invention provides substantially pure proteins or peptides comprising sequences corresponding to at least 4, preferably at least 9, more preferably at least 15 consecutive amino acids of the CalDAG-GEF and/or cAMP-GEF proteins, as disclosed or otherwise enabled herein.

Purification can be achieved using standard protein purification procedures including, but not limited to, gel-filtration chromatography, ion-exchange chromatography, high-performance

- 27 -

liquid chromatography (RP-HPLC, ion-exchange HPLC, size-exclusion HPLC, high-performance chromatofocusing chromatography, hydrophobic interaction chromatography, immunoprecipitation, or immunoaffinity purification. Gel electrophoresis (*e.g.*, PAGE, SDS-PAGE) can also be used to isolate a protein or peptide based on its molecular weight, charge properties, and hydrophobicity.

A CalDAG-GEF or cAMP-GEF protein, or a fragment thereof, may also be conveniently purified by creating a fusion protein including the desired CalDAG-GEF or cAMP-GEF sequence fused to another peptide such as an antigenic determinant or poly-His tag (*e.g.*, QIAexpress vectors, (QIAGEN Corp., Chatsworth, CA)), or a larger protein (*e.g.*, GST using the pGEX-27 vector (Amrad, USA) or green fluorescent protein using the Green Lantern vector (GIBCO/BRL, Gaithersburg, MD)).

### 3. Antibodies to the CalDAG-GEF and/or cAMP-GEFs

The present invention also provides antibodies, and methods of making antibodies, which selectively bind to the CalDAG-GEF and/or cAMP-GEF proteins or fragments thereof. The antibodies of the invention have utility as laboratory reagents for, *inter alia*, immunoaffinity purification of the CalDAG-GEFs and/or cAMP-GEFs, Western blotting to identify cells or tissues expressing the CalDAG-GEFs and/or cAMP-GEFs, and immunocytochemistry or immunofluorescence techniques to establish the subcellular location of the protein.

The antibodies of the invention may be generated in a host using the entire CalDAG-GEF and/or cAMP-GEF proteins of the invention or using any CalDAG-GEF and/or cAMP-GEF epitope which is characteristic of that protein and which substantially distinguishes it from host proteins. Such epitopes may be identified by comparing sequences of, for example, 4-8 amino acid residues from a CalDAG-GEF and/or cAMP-GEF sequence to computer databases of protein sequences from the relevant host. Antibodies against highly conserved domains are expected to have the greatest utility for purification or identification of CalDAG-GEFs and/or cAMP-GEFs.

Amino acid residue positions which are potential antigenic sites in the CalDAG-GEF or cAMP-GEF proteins and which may be useful in generating the antibodies of the invention may be determined by using computer programs such as the IBI Pustell program. Other methods of choosing antigenic determinants are known in the art and may, of course, be employed. In addition, larger fragments (*e.g.*, 9-15 residues) including some of these epitopes may also be employed. Even larger fragments, including, for example, entire functional domains or multiple

- 28 -

functional domains may also be preferred. For an overview of antibody techniques, see Antibody Engineering: A Practical Guide, Borrebaek, ed., W.H. Freeman & Company, NY (1992), or Antibody Engineering, 2nd Ed., Borrebaek, ed., Oxford University Press, Oxford (1995).

The antibodies of the invention may be labelled or conjugated with other compounds or materials for diagnostic and/or therapeutic uses. For example, they may be coupled to radionuclides, fluorescent compounds, or enzymes for imaging or therapy, or to liposomes for the targeting of compounds contained in the liposomes to a specific tissue location.

#### 4. Transformed Cell Lines

The present invention also provides for cells or cell lines, both prokaryotic and eukaryotic, which have been transformed or transfected with the nucleic acids of the present invention so as to cause clonal propagation of those nucleic acids and/or expression of the proteins or peptides encoded thereby. Such cells or cell lines will have utility not only in the propagation and production of the nucleic acids and proteins of the present invention but also, as further described herein, as model systems for diagnostic and therapeutic assays. As used herein, the term "transformed cell" is intended to embrace any cell, or the descendant of any cell, into which has been introduced any of the nucleic acids of the invention, whether by transformation, transfection, infection, electroporation, microinjection or other means. Methods of producing appropriate vectors, transforming cells with those vectors, and identifying transformants are well known in the art and are only briefly reviewed here (see, for example, Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

Vectors may be introduced into the recipient or "host" cells by various methods well known in the art including, but not limited to, calcium phosphate transfection, strontium phosphate transfection, DEAE dextran transfection, electroporation, lipofection (e.g., Dosper Liposomal transfection reagent, Boehringer Mannheim, Germany), microinjection, ballistic insertion on micro-beads, protoplast fusion or, for viral or phage vectors, by infection with the recombinant virus or phage.

#### 5. Transgenic Animal Models

The present invention also provides for the production of transgenic non-human animal models for the study of Ras-related cancers, for the screening of candidate pharmaceutical compounds, for the creation of explanted mammalian cell cultures (e.g., neuronal, glial,



organotypic or mixed cell cultures) in which mutant or wild type CalDAG-GEF and/or cAMP-GEF sequences are expressed or in which the CalDAG-GEF and/or cAMP-GEF genes have been inactivated (*e.g.*, "knock-out" deletions), and for the evaluation of potential therapeutic interventions.

5 Species suitable for use as animal models in the present invention include, but are not limited to, rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates (*e.g.*, Rhesus monkeys, chimpanzees).

Various techniques for generating transgenic animals, as well as techniques for homologous recombination or gene targeting, are now widely accepted and practiced. See, for  
10 example, Hogan et al., Manipulating Mouse Embryo (1986). To create a transgene, the target sequence of interest (*e.g.*, mutant or wild-type CalDAG-GEF or cAMP-GEF sequences) is typically ligated into a cloning site located downstream of a promoter element which will regulate the expression of RNA from the CalDAG-GEF or cAMP-GEF sequence. An alternate approach to creating a transgene is to use endogenous CalDAG-GEF or cAMP-GEF regulatory  
15 sequences to drive expression of the CalDAG-GEF or cAMP-GEF transgene.

6. Assays for Drugs Which Affect CalDAG-GEF and/or cAMP-GEF Expression

In another series of embodiments, the present invention provides assays for identifying small molecules or other compounds which are capable of inducing or inhibiting the expression of the CalDAG-GEF or cAMP-GEF genes and proteins. The assays may be performed *in vitro*  
20 using non-transformed cells, immortalized cell lines, or recombinant cell lines, or *in vivo* using the transgenic animal models enabled herein.

In particular, the assays may detect the presence of increased or decreased expression of CalDAG-GEF, cAMP-GEF, or other CalDAG-GEF or cAMP-GEF-related genes or proteins, on the basis of increased or decreased mRNA expression (using, *e.g.*, the nucleic acid probes  
25 disclosed and enabled herein), increased or decreased levels of CalDAG-GEF, cAMP-GEF or other CalDAG-GEF or cAMP-GEF-related protein products (using, *e.g.*, the anti-CalDAG-GEF or anti-cAMP-GEF antibodies disclosed and enabled herein), or increased or decreased levels of expression of a marker gene (*e.g.*,  $\beta$ -galactosidase or luciferase) operably joined to a CalDAG-GEF or cAMP-GEF 5' regulatory region in a recombinant construct.

30 Thus, for example, one may culture cells known to express a particular CalDAG-GEF or cAMP-GEF and add to the culture medium one or more test compounds. After allowing a sufficient period of time (*e.g.*, 0-72 hours) for the compound to induce or inhibit the expression

- 30 -

of the CalDAG-GEF or cAMP-GEF, any change in levels of expression from an established baseline may be detected using any of the techniques described above and well known in the art. In particularly preferred embodiments, the cells are from an immortalized cell line such as a human neuroblastoma, glioblastoma or a hybridoma cell line. Using the nucleic acid probes and/or antibodies disclosed and enabled herein, detection of changes in the expression of a CalDAG-GEF or cAMP-GEF and thus, identification of the compound as an inducer or repressor of CalDAG-GEF and/or cAMP-GEF expression, requires only routine experimentation.

In particularly preferred embodiments, a recombinant assay is employed in which a reporter gene such as a  $\beta$ -galactosidase, green fluorescent protein, alkaline phosphatase, or luciferase is operably joined to a 5' regulatory region of a CalDAG-GEF or cAMP-GEF gene. The reporter gene and regulatory regions are joined in-frame (or in each of the three possible reading frames) so that transcription and translation of the reporter gene may proceed under the control of the CalDAG-GEF or cAMP-GEF regulatory elements. The recombinant construct may then be introduced into any appropriate cell type, although mammalian cells are preferred, and human cells are most preferred. The transformed cells may be grown in culture and, after establishing the baseline level of expression of the reporter gene, test compounds may be added to the medium. The ease of detection of the expression of the reporter gene provides for a rapid, high through-put assay for the identification of inducers and repressors of the CalDAG-GEF or cAMP-GEF gene.

Compounds identified by this method will have potential utility in modifying the expression of the CalDAG-GEF, cAMP-GEF or other CalDAG-GEF or cAMP-GEF-related genes *in vivo*. These compounds may be further tested in the animal models disclosed and enabled herein to identify those compounds having the most potent *in vivo* effects. In addition, as described herein with respect to small molecules having CalDAG-GEF or cAMP-GEF-binding activity, these molecules may serve as "lead compounds" for the further development of pharmaceuticals by, for example, subjecting the compounds to sequential modifications, molecular modeling, and other routine procedures employed in rational drug design.

#### 7. Identification of Compounds with CalDAG-GEF and/or cAMP-GEF Binding Capacity

In light of the present disclosure, one of ordinary skill in the art is enabled to practice new screening methodologies which will be useful in the identification of proteins and other compounds which bind to, or otherwise directly interact with, the CalDAG-GEFs or cAMP-GEFs. The proteins and compounds will include endogenous cellular components which interact

- 31 -

with the CalDAG-GEFs or cAMP-GEFs *in vivo* and which, therefore, provide new targets for pharmaceutical and therapeutic interventions, as well as recombinant, synthetic and otherwise exogenous compounds which may have CalDAG-GEF or cAMP-GEF binding capacity and, therefore, may be candidates for pharmaceutical agents. Thus, in one series of embodiments, cell  
5 lysates or tissue homogenates (*e.g.*, human brain homogenates, leukocyte lysates) may be screened for proteins or other compounds which bind to one of the normal or mutant CalDAG-GEFs and/or cAMP-GEFs. Alternatively, any of a variety of exogenous compounds, both naturally occurring and/or synthetic (*e.g.*, libraries of small molecules or peptides), may be screened for CalDAG-GEF or cAMP-GEF binding capacity. Small molecules are particularly  
10 preferred in this context because they are more readily absorbed after oral administration, have fewer potential antigenic determinants, and/or are more likely to cross the blood brain barrier than larger molecules such as nucleic acids or proteins. The methods of the present invention are particularly useful in that they may be used to identify molecules which selectively or preferentially bind to a mutant form of a CalDAG-GEF or cAMP-GEF protein (rather than a  
15 normal form) and, therefore, may have particular utility in treating the heterozygous victims of a CalDAG-GEF or cAMP-GEF associated disorder.

Compounds which bind to normal, mutant or both forms of the CalDAG-GEFs or cAMP-GEFs may have utility in treatments and diagnostics. Compounds which bind only to a normal CalDAG-GEF or cAMP-GEF may, for example, act as enhancers of its normal activity and  
20 thereby at least partially compensate for the lost or abnormal activity of mutant forms of the CalDAG-GEF or cAMP-GEF in victims suffering from CalDAG-GEF- or cAMP-GEF-associated disorders. Compounds which bind to both normal and mutant forms of a CalDAG-GEF or cAMP-GEF may have utility if they differentially affect the activities of the two forms so as to alleviate the overall departure from normal function. Alternatively, blocking the activity of  
25 both normal and mutant forms of either CalDAG-GEF or cAMP-GEF may have less severe physiological and clinical consequences than the normal progress of the disorder and, therefore, compounds which bind to and inhibit the activity of both normal and mutant forms of a CalDAG-GEF or cAMP-GEF may be therapeutically useful. Preferably, however, compounds are identified which have a higher affinity of binding to mutant CalDAG-GEF or cAMP-GEF  
30 than to normal CalDAG-GEF or cAMP-GEF, and which selectively or preferentially inhibit the activity of the mutant form. Such compounds may be identified by using any of the techniques

- 32 -

described herein, and then comparing the binding affinities of the candidate compound(s) for the normal and mutant forms of CalDAG-GEF or cAMP-GEF.

The effect of agents which bind to the CalDAG-GEFs or cAMP-GEFs (normal or mutant forms of either) can be monitored either by direct monitoring of this binding (*e.g.*, using the BIAcore assay, LKB Pharmacia, Sweden) or by indirect monitoring of binding by detecting, for example, a change in fluorescence, molecular weight, or concentration of either the binding agent or CalDAG-GEF or cAMP-GEF component, either in a soluble phase or in a substrate-bound phase.

Once identified by the methods described above, the candidate compounds may then be produced in quantities sufficient for pharmaceutical administration or testing (*e.g.*,  $\mu\text{g}$  or  $\text{mg}$  or greater quantities), and formulated in a pharmaceutically acceptable carrier (see, *e.g.*, REMINGTON'S PHARMACEUTICAL SCIENCES, Gennaro, A., ed., Mack Pub., (1990)). These candidate compounds may then be administered to the transformed cells of the invention, to the transgenic animal models of the invention, to cell lines derived from the animal models or from human patients, or to patients with CalDAG-GEF- or cAMP-GEF-associated disorders. The animal models described and enabled herein are of particular utility in further testing candidate compounds which bind to normal or mutant CalDAG-GEF or cAMP-GEF for their therapeutic efficacy.

In addition, once identified by the methods described above, the candidate compounds may also serve as "lead compounds" in the design and development of new pharmaceuticals. For example, as is well known in the art, sequential modification of small molecules (*e.g.*, amino acid residue replacement for peptides; functional group replacement for peptide or non-peptide compounds) is a standard approach in the pharmaceutical industry for the development of new pharmaceuticals. Such development generally proceeds from a "lead compound" which is shown to have at least some of the activity (*e.g.*, CalDAG-GEF or cAMP-GEF binding or blocking ability) of the desired pharmaceutical. In particular, when one or more compounds having at least some activity of interest (*e.g.*, modulation of CalDAG-GEF or cAMP-GEF activity) are identified, structural comparison of the molecules can greatly inform the skilled practitioner by suggesting portions of the lead compounds which should be conserved and portions which may be varied in the design of new candidate compounds. Thus, the present invention also provides a means of identifying lead compounds which may be sequentially modified to produce new candidate compounds for use in the treatment of CalDAG-GEF- or cAMP-GEF-associated

- 33 -

disorders. These new compounds then may be tested both for CalDAG-GEF or cAMP-GEF-binding or blocking (*e.g.*, in the binding assays described above) and for therapeutic efficacy (*e.g.*, in the animal models described herein). This procedure may be iterated until compounds having the desired therapeutic activity and/or efficacy are identified.

5 In each of the present series of embodiments, an assay is conducted to detect binding between a "CalDAG-GEF component" or a "cAMP-GEF component" and some other moiety. Of particular utility will be sequential assays in which compounds are tested for the ability to bind to only the normal or only the mutant forms of the CalDAG-GEF or cAMP-GEF functional domains using mutant and normal CalDAG-GEF or cAMP-GEF components in the binding  
10 assays. Such compounds are expected to have the greatest therapeutic utilities, as described more fully below. The "CalDAG-GEF component" or the "cAMP-GEF component" in these assays may be a complete normal or mutant form of a CalDAG-GEF or cAMP-GEF protein (*e.g.*, an hCalDAG-GEF or hcAMP-GEF variant) but need not be. Rather, particular functional domains of the CalDAG-GEFs or cAMP-GEFs, as described above, may be employed either as  
15 separate molecules or as part of a fusion protein. For example, to isolate proteins or compounds that interact with these functional domains, screening may be carried out using fusion constructs and/or synthetic peptides corresponding to these regions. Obviously, various combinations of fusion proteins and functional domains from CalDAG-GEF or cAMP-GEF are possible. In addition, the functional domains may be altered so as to aid in the assay by, for example,  
20 introducing into the functional domain a reactive group or amino acid residue (*e.g.*, cysteine) which will facilitate immobilization of the domain on a substrate (*e.g.*, using sulfhydryl reactions).

Methods for screening cellular lysates, tissue homogenates, or small molecule libraries for candidate CalDAG-GEF or cAMP-GEF-binding molecules are well known in the art and, in  
25 light of the present disclosure, may now be employed to identify compounds which bind to normal or mutant CalDAG-GEF or cAMP-GEF components or which modulate CalDAG-GEF or cAMP-GEF activity as defined by non-specific measures (*e.g.*, changes in intracellular  $\text{Ca}^{2+}$ , GTP/GDP ratio) or by specific measures (*e.g.*, changes in the expression of other downstream genes which can be monitored by differential display, 2D gel electrophoresis, differential  
30 hybridization, or SAGE methods). The preferred methods involve variations on the following techniques: (1) direct extraction by affinity chromatography; (2) co-isolation of CalDAG-GEF or cAMP-GEF components and bound proteins or other compounds by immunoprecipitation; (3)

- 34 -

the Biomolecular Interaction Assay (BIAcore); and (4) the yeast two-hybrid systems. These and others are discussed separately below.

#### A. Affinity Chromatography

In light of the present disclosure, a variety of affinity binding techniques well known in the art may be employed to isolate proteins or other compounds which bind to the CalDAG-GEFs or cAMP-GEFs disclosed or otherwise enabled herein. In general, a CalDAG-GEF or cAMP-GEF component may be immobilized on a substrate (*e.g.*, a column or filter) and a solution including the test compound(s) is contacted with the CalDAG-GEF or cAMP-GEF protein, fusion or fragment under conditions which are permissive for binding. The substrate is then washed with a solution to remove unbound or weakly bound molecules. A second wash may then elute those compounds which strongly bound to the immobilized normal or mutant CalDAG-GEF or cAMP-GEF component. Alternatively, the test compounds may be immobilized and a solution containing one or more CalDAG-GEF or cAMP-GEF components may be contacted with the column, filter, or other substrate. The ability of the CalDAG-GEF or cAMP-GEF component to bind to the test compounds may be determined as above or a labeled form of the CalDAG-GEF or cAMP-GEF component (*e.g.*, a radio-labeled or chemiluminescent functional domain) may be used to more rapidly assess binding to the substrate-immobilized compound(s).

#### B. Co-Immunoprecipitation

Another well characterized technique for the isolation of the CalDAG-GEF or cAMP-GEF components and their associated proteins or other compounds is direct immunoprecipitation with antibodies. This procedure has been successfully used, for example, to isolate many of the synaptic vesicle associated proteins (Phizicky et al., 59 J. BIOL. CHEM. 94-123 (1994)). Thus, either normal or mutant CalDAG-GEF or cAMP-GEF components may be mixed in a solution with the candidate compound(s) under conditions which are permissive for binding, and the CalDAG-GEF or cAMP-GEF component may be immunoprecipitated. Proteins or other compounds which co-immunoprecipitate with the CalDAG-GEF or cAMP-GEF component may then be identified by standard techniques as described above. General techniques for immunoprecipitation may be found in, for example, Harlow et al., ANTIBODIES: A LABORATORY MANUAL (1988).

- 35 -

The antibodies employed in this assay, as described and enabled herein, may be polyclonal or monoclonal, and include the various antibody fragments as well as single chain antibodies, and the like.

C. The Biomolecular Interaction Assay

5 Another useful method for the detection and isolation of binding proteins is the Biomolecular Interaction Assay or "BIAcore" system developed by Pharmacia Biosensor and described in the manufacturer's protocol (LKB Pharmacia, Sweden). In light of the present disclosure, one of ordinary skill in the art is now enabled to employ this system, or a substantial equivalent, to identify proteins or other compounds having CalDAG-GEF or cAMP-GEF binding  
10 capacity. The BIAcore system uses an affinity purified anti-GST antibody to immobilize GST-fusion proteins onto a sensor chip. Obviously, other fusion proteins and corresponding antibodies may be substituted. The sensor utilizes surface plasmon resonance which is an optical phenomenon that detects changes in refractive indices. A homogenate of a tissue of interest is passed over the immobilized fusion protein and protein-protein interactions are registered as  
15 changes in the refractive index. This system can be used to determine the kinetics of binding and to assess whether any observed binding is of physiological relevance.

D. The Yeast Two-Hybrid System

The yeast "two-hybrid" system takes advantage of transcriptional factors that are composed of two physically separable, functional domains. One commonly used system employs  
20 the yeast GAL4 transcriptional activator, consisting of a DNA binding domain and a transcriptional activation domain. Two different cloning vectors are used to generate separate fusions of the GAL4 domains to genes encoding potential binding proteins. The fusion proteins are co-expressed, targeted to the nucleus and, if interactions occur, activation of a reporter gene (*e.g.*, lacZ) produces a detectable phenotype.

25 E. Other Methods

The nucleotide sequences and protein products, including both mutant and normal forms of these nucleic acids and their corresponding proteins, can be used with the above techniques to isolate other interacting proteins, and to identify other genes whose expression is altered by the over-expression of normal CalDAG-GEF or cAMP-GEF sequences, by the under-expression of  
30 normal CalDAG-GEFs or cAMP-GEFs sequences, or by the expression of mutant CalDAG-GEF and/or cAMP-GEF sequences. Identification of these interacting proteins, as well as the identification of other genes whose expression levels are altered in the face of mutant CalDAG-

- 36 -

GEF or cAMP-GEF sequences (for instance) will identify other gene targets which have direct relevance to the pathogenesis of this disease in its clinical or pathological forms. Specifically, these techniques rely on PCR-based and/or hybridization-based methods to identify genes which are differentially expressed between two conditions (a cell line expressing normal CalDAG-GEFs or cAMP-GEFs compared to the same cell type expressing a mutant CalDAG-GEF or cAMP-GEF sequence). These techniques include differential display, serial analysis of gene expression (SAGE), mass-spectrometry of protein, 2D-gels and subtractive hybridization (*See, e.g.,* Nowak, 270 Sci. 368-371 (1995); Kahn, 270 Sci. 369-370 (1995)).

8. Methods of Identifying Compounds Modulating CalDAG-GEF and/or cAMP-GEF Activity

In another series of embodiments, the present invention provides for methods of identifying compounds with the ability to modulate the activity of normal and mutant CalDAG-GEFs and/or cAMP-GEFs. As used with respect to this series of embodiments, the term "activity" broadly includes gene and protein expression, CalDAG-GEF and/or cAMP-GEF protein post-translation processing, trafficking and localization, and any functional activity (*e.g.,* enzymatic, receptor-effector, binding, channel), as well as downstream affects of any of these. Using the transformed cells and transgenic animal models of the present invention, cells obtained from subjects bearing a mutant CalDAG-GEF and/or cAMP-GEF gene, or animals or human subjects bearing naturally occurring CalDAG-GEF and/or cAMP-GEF mutations, it is now possible to screen candidate pharmaceuticals and treatments for their therapeutic effects by detecting changes in one or more of the functional characteristics or phenotypic manifestations of normal or mutant CalDAG-GEF and/or cAMP-GEF expression.

Thus, the present invention provides methods for screening or assaying for proteins, small molecules or other compounds which modulate CalDAG-GEF and/or cAMP-GEF activity by contacting a cell *in vivo* or *in vitro* with a candidate compound and assaying for a change in a marker associated with normal or mutant CalDAG-GEF and/or cAMP-GEF activity. The marker associated with CalDAG-GEF and/or cAMP-GEF activity may be any measurable biochemical, physiological, histological and/or behavioral characteristic associated with CalDAG-GEF and/or cAMP-GEF expression. In particular, useful markers will include any measurable biochemical, physiological, histological and/or behavioral characteristic which distinguishes cells, tissues, animals or individuals bearing at least one mutant CalDAG-GEF and/or cAMP-GEF gene from their normal counterparts. In addition, the marker may be any specific or non-specific measure of CalDAG-GEF and/or cAMP-GEF activity, such as the GDP/GTP bound to Rap1/Ras.



- 37 -

CalDAG-GEF and/or cAMP-GEF specific measures include measures of CalDAG-GEF and/or cAMP-GEF expression (*e.g.*, CalDAG-GEF and/or cAMP-GEF mRNA or protein levels) which may employ the nucleic acid probes or antibodies of the present invention. Non-specific measures include changes in cell physiology such as pH, intracellular calcium, cAMP levels, overall GTP/GDP ratios, phosphatidylinositol activity, protein phosphorylation, etc.; which can be monitored by known methods. The activation or inhibition of CalDAG-GEF or cAMP-GEF activity in its mutant or normal form can also be monitored by examining changes in the expression of other genes which are specific to the CalDAG-GEF and/or cAMP-GEF pathway. These can be assayed by such techniques as differential display, differential hybridization, and SAGE, as well as by 2-D gel electrophoresis of cellular lysates. In each case, the differentially-expressed genes can be ascertained by inspection of identical studies before and after application of the candidate compound. Furthermore, as noted elsewhere, the particular genes whose expression is modulated by the administration of the candidate compound can be ascertained by cloning, nucleotide sequencing, amino acid sequencing, or mass spectrometry.

In general, a cell may be contacted with a candidate compound and, after an appropriate period (*e.g.*, 0-72 hours for most biochemical measures of cultured cells), the marker of CalDAG-GEF or cAMP-GEF activity may be assayed and compared to a baseline measurement. The baseline measurement may be made prior to contacting the cell with the candidate compound or may be an external baseline established by other experiments or known in the art. The cell may be a transformed cell of the present invention or an explant from an animal or individual. In particular, the cell may be an explant from a carrier of a CalDAG-GEF or cAMP-GEF mutation or an animal model of the invention (*e.g.*, a transgenic nematode or mouse bearing a mutant CalDAG-GEF or cAMP-GEF gene). Preferred cells include those from neurological tissues such as neuronal, glial or mixed cell cultures; and cultured fibroblasts, liver, kidney, spleen, or bone marrow. The cells may be contacted with the candidate compounds in a culture *in vitro* or may be administered *in vivo* to a live animal or human subject. For live animals or human subjects, the test compound may be administered orally or by any parenteral route suitable to the compound. For clinical trials of human subjects, measurements may be conducted periodically (*e.g.*, daily, weekly or monthly) for several months or years.

In light of the identification, characterization, and disclosure herein of the CalDAG-GEF or cAMP-GEF genes and proteins, the CalDAG-GEF or cAMP-GEF nucleic acid probes and antibodies, and the CalDAG-GEF or cAMP-GEF transformed cells and transgenic animals of the

- 38 -

invention, one of ordinary skill in the art is now enabled by perform a great variety of assays which will detect the modulation of CalDAG-GEF or cAMP-GEF activity by candidate compounds. Particularly preferred and contemplated embodiments are discussed in some detail below.

5       A. CalDAG-GEF and/or cAMP-GEF Expression

In one series of embodiments, specific measures of CalDAG-GEF or cAMP-GEF expression are employed to screen candidate compounds for their ability to affect CalDAG-GEF or cAMP-GEF activity. Thus, using the CalDAG-GEF or cAMP-GEF nucleic acids and antibodies disclosed and otherwise enabled herein, one may use mRNA levels or protein levels  
10 as a marker for the ability of a candidate compound to modulate CalDAG-GEF or cAMP-GEF activity. The use of such probes and antibodies to measure gene and protein expression is well known in the art and discussed elsewhere herein.

B. Intracellular Localization

In another series of embodiments, compounds may be screened for their ability to  
15 modulate the activity of the CalDAG-GEFs or cAMP-GEFs based upon their effects on the trafficking and intracellular localization of the CalDAG-GEFs or cAMP-GEFs. Differences in localization of mutant and normal CalDAG-GEFs and/or cAMP-GEFs may contribute to the etiology of CalDAG-GEF and/or cAMP-GEF-associated diseases. Compounds which can affect the localization of the CalDAG-GEFs and/or cAMP-GEFs may, therefore, be identified as  
20 potential therapeutics. Standard techniques known in the art may be employed to detect the localization of the CalDAG-GEFs and/or cAMP-GEFs. Generally, these techniques will employ the antibodies of the present invention, and in particular antibodies which selectively bind to one or more mutant CalDAG-GEFs or cAMP-GEFs but not to normal CalDAG-GEFs or cAMP-GEFs. As is well known in the art, such antibodies may be labeled by any of a variety of  
25 techniques (e.g., fluorescent or radioactive tags, labeled secondary antibodies, avidin-biotin, etc.) to aid in visualizing the intracellular location of the CalDAG-GEFs or cAMP-GEFs. The CalDAG-GEFs or cAMP-GEFs may be co-localized to particular structures, as is known in the art, using antibodies to markers of those structures (e.g., TGN38 for the Golgi, transferrin receptor for post-Golgi transport vesicles, LAMP2 for lysosomes). Western blots of purified  
30 fractions from cell lysates enriched for different intracellular membrane bound organelles (e.g., lysosomes, synaptosomes, Golgi) may also be employed. In addition, the relative orientation of

different domains of the CalDAG-GEFs and/or cAMP-GEFs across cellular domains may be assayed using, for example, electron microscopy and antibodies raised to those domains.

9. Screening and Diagnostics for CalDAG-GEF- or cAMP-GEF-associated disorders

A. General Diagnostic Methods

5       The CalDAG-GEF or cAMP-GEF genes and gene products, as well as the CalDAG-GEF or cAMP-GEF-derived probes, primers and antibodies, disclosed or otherwise enabled herein, are useful in the screening for carriers of alleles associated with CalDAG-GEF- or cAMP-GEF-associated disorders. Individuals at risk for such a disorder or individuals not previously known to be at risk, may be routinely screened using probes to detect the presence of a mutant CalDAG-GEF or cAMP-GEF gene or protein by a variety of techniques. Diagnosis of inherited cases of these diseases can be accomplished by methods based upon the nucleic acids (including genomic and mRNA/cDNA sequences), proteins, and/or antibodies disclosed and enabled herein, including functional assays designed to detect increases or decreases of the normal CalDAG-GEF or cAMP-GEF activity and/or the presence of specific new activities conferred by the mutant CalDAG-GEFs or cAMP-GEFs. Preferably, the methods and products are based upon the human CalDAG-GEF or cAMP-GEF nucleic acids, proteins or antibodies, as disclosed or otherwise enabled herein. For brevity of exposition, but without limiting the scope of the invention, the following description will focus upon uses of the human homologues of CalDAG-GEF and cAMP-GEF. It will be understood, however, that homologous sequences from other species, including those disclosed herein, will be equivalent for many purposes.

B. Protein Based Screens and Diagnostics

When a diagnostic assay is to be based upon CalDAG-GEF or cAMP-GEF proteins, a variety of approaches are possible. For example, diagnosis can be achieved by monitoring differences in the electrophoretic mobility of normal and mutant proteins. Such an approach will be particularly useful in identifying mutants in which insertions, deletions or substitutions have resulted in a significant change in the electrophoretic migration of the resultant protein. Alternatively, diagnosis may be based upon differences in the proteolytic cleavage patterns of normal and mutant proteins, differences in molar ratios of the various amino acid residues, or by functional assays demonstrating altered function of the gene products.

C. Nucleic Acid Based Screens and Diagnostics

When the diagnostic assay is to be based upon nucleic acids from a sample, the assay may be based upon mRNA, cDNA or genomic DNA. Whether mRNA, cDNA, or genomic DNA is

- 40 -

assayed, standard methods well known in the art may be used to detect the presence of a particular sequence either *in situ* or *in vitro* (See, e.g., Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed. (1989)).

(1) Appropriate Probes and Primers

5 Whether for hybridization, RNase protection, ligase-mediated detection, PCR amplification or any other standards methods described herein and well known in the art, a variety of subsequences of the CalDAG-GEF and/or cAMP-GEF sequences disclosed or otherwise enabled herein will be useful as probes and/or primers. These sequences or subsequences will include both normal CalDAG-GEF or cAMP-GEF sequences and deleterious  
10 mutant sequences. In general, useful sequences will include at least 8-10, more preferably 10-15, and most preferably 15-25 consecutive nucleotides from the CalDAG-GEF or cAMP-GEF introns, exons or intron/exon boundaries. In another embodiment, useful sequences include at least 25-500 consecutive nucleotides. Depending upon the target sequence, the specificity required, and future technological developments, shorter sequences may also have utility.  
15 Therefore, any CalDAG-GEF or cAMP-GEF derived sequence which is employed to isolate, clone, amplify, identify or otherwise manipulate a CalDAG-GEF or cAMP-GEF sequence may be regarded as an appropriate probe or primer.

(2) Hybridization Screening

For *in situ* detection of a normal or mutant CalDAG-GEF, cAMP-GEF or other CalDAG-  
20 GEF and/or cAMP-GEF-associated nucleic acid sequence, a sample of tissue may be prepared by standard techniques and then contacted with one or more of the above-described probes, preferably one which is labeled to facilitate detection, and an assay for nucleic acid hybridization is conducted under stringent conditions which permit hybridization only between the probe and highly or perfectly complementary sequences.

25 (3) Restriction Mapping

Sequence alterations may also create or destroy fortuitous restriction enzyme recognition sites which are revealed by the use of appropriate enzyme digestion followed by electrophoresis and visualization. DNA fragments carrying the site (normal or mutant) are detected by their increase or reduction in size, or by the increase or decrease of corresponding restriction fragment  
30 numbers. Such restriction fragment length polymorphism analysis (RFLP), or restriction mapping, may be employed with genomic DNA, mRNA or cDNA. The CalDAG-GEF or cAMP-GEF sequences may be amplified by PCR using the above-described primers prior to

- 41 -

restriction, in which case the lengths of the PCR products may indicate the presence or absence of particular restriction sites, and/or may be subjected to restriction after amplification. The CalDAG-GEF or cAMP-GEF fragments may be visualized by any convenient means (*e.g.*, under UV light in the presence of ethidium bromide).

5                   (4) PCR Mapping

In another series of embodiments, a single base substitution mutation may be detected based on differential PCR product length or production in PCR. Thus, primers which span mutant sites or which, preferably, have 3' termini at mutation sites, may be employed to amplify a sample of genomic DNA, mRNA or cDNA from a subject. A mismatch at a mutational site may  
10 be expected to alter the ability of the normal or mutant primers to promote the polymerase reaction and, thereby, result in product profiles which differ between normal subjects and heterozygous and/or homozygous CalDAG-GEF or cAMP-GEF mutants.

(5) Electrophoretic Mobility

Genetic testing based on DNA sequence differences also may be achieved by detection of  
15 alterations in electrophoretic mobility of DNA, mRNA or cDNA fragments in gels. Small sequence deletions and insertions, for example, can be visualized by high resolution gel electrophoresis of single or double stranded DNA, or as changes in the migration pattern of DNA heteroduplexes in non-denaturing gel electrophoresis.

(6) Chemical Cleavage of Mismatches

20 Mutations in the CalDAG-GEFs or cAMP-GEFs may also be detected by employing the chemical cleavage of mismatch (CCM) method (*See, e.g.*, Saleeba et al., METHODS IN ENZYMOLOGY, 217: 286-295 (1993)). In this technique, probes (up to ~ 1 kb) may be mixed with a sample of genomic DNA, cDNA or mRNA obtained from a subject. The sample and probes are mixed and subjected to conditions which allow for heteroduplex formation (if any).  
25 Preferably, both the probe and sample nucleic acids are double-stranded, or the probe and sample may be PCR amplified together, to ensure creation of all possible mismatch heteroduplexes. Mismatched T residues are reactive to osmium tetroxide and mismatched C residues are reactive to hydroxylamine. Because each mismatched A will be accompanied by a mismatched T, and each mismatched G will be accompanied by a mismatched C, any nucleotide differences between  
30 the probe and sample (including small insertions or deletions) will lead to the formation of at least one reactive heteroduplex. After treatment with osmium tetroxide and/or hydroxylamine to modify any mismatch sites, the mixture is subjected to chemical cleavage at any modified

- 42 -

mismatch sites by, for example, reaction with piperidine. The mixture may then be analyzed by standard techniques such as gel electrophoresis to detect cleavage products which would indicate mismatches between the probe and sample.

#### (7) Other Methods

5 Various other methods of detecting CalDAG-GEF or cAMP-GEF mutations, based upon the CalDAG-GEF or cAMP-GEF sequences disclosed and otherwise enabled herein, will be apparent to those of ordinary skill in the art. Any of these may be employed in accordance with the present invention. These include, but are not limited to, nuclease protection assays (S1 or ligase-mediated), ligated PCR, denaturing gradient gel electrophoresis (DGGE; *see, e.g.*, Fischer  
10 et al., 80 PROC. NAT'L ACAD. SCI (USA), 1578-83 (1983)), restriction endonuclease fingerprinting combined with SSCP (REF-SSCP; *see, e.g.*, Liu et al., 18 BIOTECHNIQUES 470-79 (1995)), and the like.

#### D. Other Screens and Diagnostics

Diagnosis also can be made by observation of alterations in CalDAG-GEF or cAMP-GEF  
15 transcription, translation, and post-translational modification and processing as well as alterations in the intracellular and extracellular trafficking of CalDAG-GEF or cAMP-GEF gene products in the brain and peripheral cells. Such changes will include alterations in the amount of CalDAG-GEF or cAMP-GEF messenger RNA and/or protein, alteration in phosphorylation state, abnormal intracellular location/distribution, abnormal extracellular distribution, etc. Such assays  
20 will include: Northern Blots (with CalDAG-GEF or cAMP-GEF-specific and non-specific nucleotide probes), Western blots and enzyme-linked immunosorbent assays (ELISA) (with antibodies raised specifically to a CalDAG-GEF or a cAMP-GEF functional domain, including various post-translational modification states).

#### E. Screening and Diagnostic Kits

25 In accordance with the present invention, diagnostic kits are also provided which will include the reagents necessary for the above-described diagnostic screens. For example, kits may be provided which include antibodies or sets of antibodies which are specific to one or more mutant epitopes. These antibodies may, in particular, be labeled by any of the standard means which facilitate visualization of binding. Alternatively, kits may be provided in which  
30 oligonucleotide probes or PCR primers, as described above, are present for the detection and/or amplification of mutant CalDAG-GEF, cAMP-GEF or other CalDAG-GEF and/or cAMP-GEF-associated nucleotide sequences. Again, such probes may be labeled for easier detection of

- 43 -

specific hybridization. As appropriate to the various diagnostic embodiments described above, the oligonucleotide probes or antibodies in such kits may be immobilized to substrates and appropriate controls may be provided.

#### 10. Methods of Treatment

5 The present invention now provides a basis for therapeutic intervention in diseases which are associated to the CalDAG-GEFs or cAMP-GEFs in that they are caused, prevented, exacerbated, or alleviated, or which may be caused, prevented, exacerbated, or alleviated, by the either normal or mutant CalDAG-GEFs or cAMP-GEFs. In considering the various therapies described below, it is understood that such therapies may be targeted at tissue other than the brain  
10 where CalDAG-GEF or cAMP-GEF are also expressed.

Therapies to treat CalDAG-GEF and/or cAMP-GEF-associated diseases may be based upon (1) administration of normal CalDAG-GEF or cAMP-GEF proteins, (2) gene therapy with normal CalDAG-GEF or cAMP-GEF genes to compensate for or replace the mutant genes, (3) gene therapy based upon antisense sequences to mutant CalDAG-GEF or cAMP-GEF genes or  
15 which "knock-out" the mutant genes, (4) gene therapy based upon sequences which encode a protein which blocks or corrects the deleterious effects of CalDAG-GEF or cAMP-GEF mutants, (5) immunotherapy based upon antibodies to normal and/or mutant CalDAG-GEF or cAMP-GEF proteins, or (6) small molecules (drugs) which alter CalDAG-GEF or cAMP-GEF expression, block abnormal interactions between mutant forms of CalDAG-GEF or cAMP-GEF and other  
20 proteins or ligands, or which otherwise block the aberrant function of mutant CalDAG-GEF or cAMP-GEF proteins by altering the structure of the mutant proteins, by enhancing their metabolic clearance, or by inhibiting their function.

##### A. Protein Therapy

Treatment of CalDAG-GEF and/or cAMP-GEF-associated disorders, or disorders  
25 resulting from CalDAG-GEF and/or cAMP-GEF mutations, may be performed by providing an excess of inactive mutant protein to decrease the effect of the normal function of the protein, or by providing an excess of normal protein to reduce the effect of any aberrant function of the mutant protein, by replacing a mutant protein with normal protein, or by modulating the function of the mutant protein.

##### 30 B. Gene Therapy

In one series of embodiments, gene therapy may be employed in which normal or mutant copies of the CalDAG-GEF gene or the cAMP-GEF gene are introduced into patients to code

- 44 -

successfully for normal or mutant protein in one or more different affected cell types. The gene must be delivered to those cells in a form in which it can be taken up and code for sufficient protein to provide effective function. Thus, it is preferred that the recombinant gene be operably joined to a strong promoter so as to provide a high level of expression which will compensate for, or out-compete, the naturally-occurring proteins. As noted above, the recombinant construct may contain endogenous or exogenous regulatory elements, inducible or repressible regulatory elements, or tissue-specific regulatory elements.

In another series of embodiments, gene therapy may be employed to replace the naturally-occurring gene by homologous recombination with a recombinant construct. The recombinant construct may contain a normal or a mutant copy of the targeted CalDAG-GEF and/or cAMP-GEF gene, in which case the defect is corrected *in situ*, or may contain a "knock-out" construct which introduces a stop codon, missense mutation, or deletion which abolished function of the mutant gene. It should be noted in this respect that such a construct may knock-out both the normal and mutant copies of the targeted CalDAG-GEF and/or cAMP-GEF gene in a heterozygous individual, but the total loss of CalDAG-GEF and/or cAMP-GEF gene function may be less deleterious to the individual than continued progression of the disease state.

In another series of embodiments, antisense gene therapy may be employed. The antisense therapy is based on the fact that sequence-specific suppression of gene expression can be achieved by intracellular hybridization between mRNA or DNA and a complementary antisense species. The formation of a hybrid duplex may then interfere with the transcription of the gene and/or the processing, transport, translation and/or stability of the target CalDAG-GEF and/or cAMP-GEF mRNA. Antisense strategies may use a variety of approaches including the administration of antisense oligonucleotides or antisense oligonucleotide analogs (*e.g.*, analogs with phosphorothioate backbones) or transfection with antisense RNA expression vectors. Again, such vectors may include exogenous or endogenous regulatory regions, inducible or repressible regulatory elements, or tissue-specific regulatory elements.

In another series of embodiments, gene therapy may be used to introduce a recombinant construct encoding a protein or peptide which blocks or otherwise corrects the aberrant function caused by a naturally-occurring CalDAG-GEF and/or cAMP-GEF gene. In one embodiment, the recombinant gene may encode a peptide which corresponds to a mutant domain of a CalDAG-GEF and/or cAMP-GEF which has been found to abnormally interact with another cell protein or other cell ligand. Alternatively, the portion of a protein which interacts with a mutant, but not a



- 45 -

normal, CalDAG-GEF and/or cAMP-GEF may be encoded and expressed by a recombinant construct in order to compete with, and thereby inhibit or block, the aberrant interaction.

Retroviral vectors can be used for somatic cell gene therapy especially because of their high efficiency of infection and stable integration and expression. The targeted cells however must be able to divide and the expression of the levels of normal protein should be high. The full length CalDAG-GEF or cAMP-GEF genes, subsequences encoding functional domains of the CalDAG-GEFs or cAMP-GEFs, or any of the other therapeutic peptides described above, can be cloned into a retroviral vector and driven from its endogenous promoter, from the retroviral long terminal repeat, or from a promoter specific for the target cell type of interest. Other viral vectors which can be used include adeno-associated virus, vaccinia virus, bovine papilloma virus, or a herpes virus such as Epstein-Barr virus.

#### C. Immunotherapy

Antibodies may be raised to a mutant CalDAG-GEF or cAMP-GEF protein (or a portion thereof) and be administered to a patient to bind or block the mutant protein and prevent its deleterious effects. Alternatively, antibodies may be raised to specific complexes between mutant or wild-type CalDAG-GEF or cAMP-GEF and their interaction partners.

A further approach is to stimulate endogenous antibody production to the desired antigen. An immunogenic composition may be prepared as injectables, as liquid solutions or emulsions. The CalDAG-GEF or cAMP-GEF protein or other antigen may be mixed with pharmaceutically acceptable excipients compatible with the protein. Such excipients may include water, saline, dextrose, glycerol, ethanol and combinations thereof. The immunogenic composition and vaccine may further contain auxiliary substances such as emulsifying agents or adjuvants to enhance effectiveness. Immunogenic compositions and vaccines may be administered parenterally by injection subcutaneously or intramuscularly.

The immunogenic preparations and vaccines are administered in such amount as will be therapeutically effective, protective and immunogenic. Dosage depends on the route of administration and will vary according to the size of the host.

#### D. Small Molecule Therapeutics

As described and enabled herein, the present invention provides for a number of methods of identifying small molecules or other compounds which may be useful in the treatment of CalDAG-GEF- or cAMP-GEF-associated disorders. Thus, for example, the present invention provides for methods of identifying CalDAG-GEF or cAMP-GEF binding proteins and, in

- 46 -

particular, methods for identifying proteins or other cell components which bind to or otherwise interact with mutant CalDAG-GEFs or cAMP-GEFs but not with the normal CalDAG-GEFs or cAMP-GEFs. The invention also provides for methods of identifying small molecules which can be used to disrupt undesired interactions between CalDAG-GEFs or cAMP-GEFs and other proteins or other cell components.

By identifying these proteins and analyzing these interactions, it is possible to screen for or design compounds which counteract or prevent the interaction, thereby, providing treatment for abnormal interactions. Therapies can be designed to modulate these interactions and thereby, to modulate CalDAG-GEF- or cAMP-GEF-associated disorders. The potential efficacy of these therapies can be tested by analyzing the affinity and function of these interactions after exposure to the therapeutic agent by standard pharmacokinetic measurements of affinity (*e.g.*,  $K_d$ ,  $V_{max}$ ) using synthetic peptides or recombinant proteins corresponding to functional domains of the CalDAG-GEF gene, the cAMP-GEF gene or other CalDAG-GEF and/or cAMP-GEF homologues. Another method for assaying the effect of any interactions involving functional domains is to monitor changes in the intracellular trafficking and post-translational modification of the relevant genes by *in situ* hybridization, immunohistochemistry, Western blotting and metabolic pulse-chase labeling studies in the presence of, and in the absence of, the therapeutic agents. A further method is to monitor the effects of "downstream" events including changes in second messenger events, *e.g.*, cAMP, intracellular  $Ca^{2+}$ , protein kinase activities, etc.

The effect of potential therapeutic agents in cell lines and whole animals can be monitored by monitoring transcription, translation, and post-translational modification of the CalDAG-GEF and/or cAMP-GEF proteins. Methods for these studies include Western and Northern blots, immunoprecipitation after metabolic labelling (pulse-chase) with radio-labelled methionine and ATP, and immunohistochemistry. The effect of these agents can also be monitored using studies which examine the relative binding affinities and relative amounts of CalDAG-GEF or cAMP-GEF proteins involved in interactions with Rap1A, using either standard binding affinity assays or co-precipitation and Western blots using antibodies to Rap1A, CalDAG-GEF, cAMP-GEF, or other CalDAG-GEF and/or cAMP-GEF homologues.

Therapy using antisense oligonucleotides to block the expression of the mutant CalDAG-GEF gene or the mutant cAMP-GEF gene, co-ordinated with gene replacement with normal CalDAG-GEF or cAMP-GEF gene can also be applied using standard techniques of either gene therapy or protein replacement therapy.

- 47 -

## V. Examples

### Example 1: Isolation and characterization of CalDAG-GEF.

Human full-length CalDAG-GEFI cDNAs were isolated from a human frontal cortex  $\lambda$ ZAPII cDNA library (Stratagene) and a U937  $\lambda$ ZAPII cDNA library. Mouse full-length  
5 CalDAG-GEFI was identified in the mouse EST database (GenBank accession number: W71787). Rat full-length CalDAG-GEFII cDNA was isolated from a rat whole brain  $\lambda$ ZAPII cDNA library by using human CalDAG-GEFII as a probe. Mouse ESTs identified through BLAST searches were purchased from Genome Systems Inc. (St. Louis, MO).

CalDAG-GEFI encodes an approximately 69-kD protein (Fig. 2D) that displays in its  
10 amino terminal region a GEF domain that is highly homologous to Ras-superfamily GEFs (Fig. 2A-2D). Multiple alignment analysis shows that genes of the CalDAG-GEF family form a cluster within the Ras-GEF superfamily distinct from Ras GEFs such as Sos1 and rRas-GEF (Fig. 2B). The region downstream of the GEF domain contains two tandem repeats of EF-hand  $\text{Ca}^{2+}$  binding motifs (Figs. 2A, 2E). The carboxy-terminal region displays a typical  
15 diacylglycerol/phorbol ester-binding domain, which is present in most PKC family proteins (Fig. 2A, 2F). Multiple sequence alignments and phylogenetic tree analysis were carried out with the LASERGENE Software Package (DNASTAR Inc.). Abbreviations and GenBank accession numbers of the protein sequences used in Figure 2 are as follows: C3G: 474982, mCdc25: 882120, rRas-GRF: 57665, hSos1 (human son-of-sevenless 1): 476780, BUD5: 171141,  
20 hCalmodulin: 115512, hCalbindin D28k: 227666, hCalcineurin B: 105504, hParvalbumin a: 131100, hTroponin C: 136043, hPKCa: 125549, hPKCb1: 125538, hPKCg: 462455.

To determine the small G protein target of CalDAG-GEFI, guanine nucleotide exchange activity *in vivo* was analyzed using intact 293T cells cotransfected with a eukaryotic expression construct of mouse CalDAG-GEFI and GST-tagged Ras family proteins. Full-length mouse  
25 CalDAG-GEFI cDNA inserted into a pCMV-SPORT expression vector with a carboxy-terminal FLAG epitope was used for transfection. A PCR-amplified fragment of rat CalDAG-GEFII was subcloned into a pCAGGS expression vector with the addition of His<sub>6</sub>-tag at its amino-terminus, resulting in pCAGGS-His-CalDAGII. pEBG-Krev1 that expresses Rap1A was used as a fusion protein to glutathione S-transferase (GST) in mammalian cells, as described in Gotoh et al., 15  
30 Mol. Cell Biol. 6746-53 (1995), pEBG-R-Ras, other vectors for Ras-family proteins obtained by inserting PCR-amplified cDNAs into pEBG expression vector, pCAGGS-C3G and pCAGGS-MSos1, and pCEV-H-RasV12. CalDAG-GEFI transfection produced a dramatic increase in

- 48 -

GTP-bound Rap1A compared to the control but showed no or minimal activation of H-Ras, R-Ras, or Ral A. The increase in GTP-bound Rap1A was augmented in the presence of either the  $\text{Ca}^{2+}$  ionophore, A23187, or the phorbol ester, phorbol-12-myristate-13-acetate (TPA). Further, A23187 and TPA had additive effects when administered together.

5 To determine the effect of CalDAG-GEFI on the Erk/MAP kinase cascade, Elk1 activation was measured in 293T cells transfected with CalDAG-GEFI or constitutively active H-Ras (RasV12), or both. 293T cells were transfected by SuperFect (Qiagen) as described in Gotoh, *supra*, with expression vectors for GST-tagged Ras family proteins and with those for various GEFs. Cells were labeled 24 hours after transfection with  $^{32}\text{P}_i$  for 2 hr. In some  
10 experiments, cells were stimulated with either 10  $\mu\text{M}$  A23187 or 1  $\mu\text{M}$  phorbol-12-myristate-13-acetate (TPA) for 3 min. GST-tagged Ras family proteins were collected from cell lysates with glutathione Sepharose. Guanine nucleotides bound to Ras family proteins were separated by thin layer chromatography (TLC). Activation of Elk1 was examined by the PathDetect Elk1 transreporting system (Stratagene). 293T cells were transfected with pFR-Luc and pFA-Elk1  
15 with various expression vectors, and light output was detected and analyzed by the use of LAS1000 film. CalDAG-GEFI reduced RasV12 activation of Elk1 by approximately 4-fold and did not itself activate Elk1. Thus, CalDAG-GEFI strongly inhibits Ras-dependent stimulation of the Erk/MAP kinase cascade.

Northern analysis showed that human CalDAG-GEFI is expressed strongly in the brain  
20 and that CalDAG-GEFI mRNA is strikingly enriched in the striatum. Probes used included human CalDAG-GEFI: 729-bp EcoRI fragment, human CalDAG-GEFII: 584-bp SacI and HindIII fragment, rat CalDAG-GEFI: 439-bp fragment of EST clone RBC565 (GenBank accession number: C06861, and rat CalDAG-GEFII: 508-bp PCR amplified and subcloned fragment (nucleic acids 2541 to 3048 of SEQ ID NO:5). *In situ* hybridization of sections from  
25 the adult rat brain confirmed these restricted distribution patterns. Intense signal was present in the striatum (caudoputamen) and the ventral striatum (nucleus accumbens, olfactory tubercle). There was weaker signal in the olfactory bulb.

A series of monoclonal antibodies against the carboxy-terminal half of mouse CalDAG-GEFI were raised. His<sub>6</sub>-tagged mouse CalDAG-GEFI polypeptide (amino acids 349 to 608 of  
30 SEQ ID NO:1) was expressed in bacteria, purified over  $\text{Ni}^{2+}$ -nitrilotriacetic acid-agarose resin, and then used to immunize BALB/c mice. The resultant polyclonal antiserum was monitored by ELISA, Western blot, immunoprecipitation, and immunofluorescence assays on CalDAG-GEFI-

- 49 -

transfected COS-7 cells. Hybridomas were generated by PEG (polyethylene glycol)-mediated fusion of donor splenocytes to the SP2/O cell line. Positive hybridoma cell lines were identified by screening in the assays described above, and purified by limiting dilution and single-cell cloning. Three hybridoma cell lines against mouse CalDAG-GEFI (mAbs 18B11, 2D9, and 18A7), in addition to the polyclonal fusion serum, were identified. Western analysis showed that mAbs 18B11 and 2D9 were specific for CalDAG-GEFI. Lightly post-fixed, cryostat-cut 10  $\mu$ m thick sections were immunostained by the ABC (Vectastain kit) method for CalDAG-GEFI with mAbs 18B11 and 2D9 and the polyclonal fusion serum, for tyrosine hydroxylase (TH) with monoclonal antibodies from INCSTAR, and for  $\mu$  opioid receptor with polyclonal antiserum. Immunohistochemistry with mAb 18B11 showed a striking basal ganglia-enriched distribution pattern in sections of adult rat brain, with significant but weaker activity elsewhere. CalDAG-GEFI immunoreactivity marked the entire pathway from the striatal matrix compartment to the pallidum and substantia nigra pars reticulata, where very intense CalDAG-GEFI staining was present. Thus, CalDAG-GEFI is synthesized in striatal projection neurons and is transported to striatopallidal and striatonigral terminals.

To confirm that CalDAG-GEFI is synthesized in striatal projection neurons and transported to striatopallidal and striatonigral terminals in rats, intrastriatal injections of ibotenic acid (20  $\mu$ g/ $\mu$ l, 1.5  $\mu$ l per site, 5 day survival) were made unilaterally at 2 sites in the mid-lateral caudoputamen, with contralateral vehicle control injections were made. In other rats, unilateral subthalamic knife-cuts were made at an anteroposterior level between the entopeduncular nucleus and substantia nigra to sever the striatonigral efferents (1 and 3 days survivals), with control contralateral thalamic knife-cuts. These procedures all reduced CalDAG-GEFI staining in the substantia nigra. *In situ* hybridization was performed according to Simmons et al, 12 J. Histotechnol. 169-181 (1989). A 439bp rat EST clone RBC565 (98.4% identical to mouse CalDAG-GEFI nucleic acids 1777 to 2216 of SEQ ID NO:1) was isolated by BLAST search and used for making RNA probes with <sup>32</sup>P-labeled UTP (2,000 Ci/mmol, NEN, 1 Ci = 37 GBq) and T3 and T7 RNA polymerase. Brains were processed as above for CalDAG-GEFI and TH immunostaining. Thus, CalDAG-GEFI is a protein transported in striatal axons to their terminals. The terminal localization of CalDAG-GEFI was confirmed in subcellular fractionation experiments on dissected samples from the rat ventral midbrain, in which Western analysis showed the presence of CalDAG-GEFI in cytosol and in membrane fractions, including synaptosomes.

- 50 -

Because of the similarity of the GEF domains of CalDAG-GEFI and CalDAG-GEFII, the substrate specificity of CalDAG-GEFII with the same 293T cell assay system used for CalDAG-GEFI was examined. It was confirmed that CalDAG-GEFII activates Ras, and further shown that it activates H-Ras and R-Ras, but not Ral A or Rap1A. H-Ras activation was enhanced by A23187 and TPA. Moreover, CalDAG-GEFII, unlike CalDAG-GEFI, increased the transcriptional activity of Elk1 downstream to Erk/MAP kinase. Thus, in the 293T system, CalDAG-GEFI and CalDAG-GEFII target different Ras-superfamily small G proteins and have opposite effects on the MAP kinase cascade. Northern analysis further showed contrasting brain expression for CalDAG-GEFII, with highest expression being in the cerebellum, cerebral cortex, and amygdala, and low expression occurring in the striatum. Both genes are also expressed in hematopoietic organs in both human and rat.

Rap signaling is important in regulating basal ganglia output in response to  $Ca^{2+}$  and DAG. Corticostriatal inputs can activate the MAP kinase cascade in striatal projection neurons (Sgambat et al., 18 J. Neurosci. 214-26 (1993)) and phosphoinositide (PI) signaling is strongly represented in these pathways (Fotuhi et al., 13 J. Neurosci. 3300-08 (1993)). Moreover, a number of receptor systems in the striatum and its striatonigral/striatopallidal pathways are linked to  $Ca^{2+}$  and PI signaling, notably including NMDA and metabotropic glutamate receptors,  $D_2$ -class dopamine receptors, and tachykinin receptors (Fiorillo et al., 394 Nature 78-82 (1998)). A previously unrecognized signaling target for some of these systems is likely to be Rap1, via CalDAG-GEFI. In addition, CalDAG-GEFI has a synaptic function as demonstrated by the heavy accumulation of CalDAG-GEFI in the target nuclei of striatal outputs and the localization of Rap1 in synaptosomes and synaptic vesicles. The particular basal ganglia projection systems are enriched in CalDAG-GEFI and are differentially vulnerable to neurodegeneration in Huntington's disease.

Rap and Ras functions can be regulated coordinately or disjunctively by  $Ca^{2+}$  and DAG in the brain and hematopoietic organs, depending on the relative expression of CalDAG-GEFI and CalDAG-GEFII. In neurons, Ras/MAP kinase signaling has been directly implicated in synaptic transmission and the neuroplasticity underlying learning and memory. Different CalDAG-GEFI and CalDAG-GEFII expression patterns in the brain influence region-specific neuroplasticity mediated by  $Ca^{2+}$  and DAG signaling pathways. The presence of CalDAG-GEFI and CalDAG-GEFII in the hematopoietic system demonstrates the direct input of  $Ca^{2+}$  and DAG to Ras/Rap regulation of normal growth and differentiation as well as malignant transformation.

Example 2: Isolation and characterization of cAMP-GEFs.

cAMP-GEFI and cAMP-GEFII have similar domain structures, with a cAMP binding domain at the amino terminus and a GEF domain at the carboxy terminus separated by a link region (LR) (Fig. 3A). These mammalian proteins show strong structural homology to a predicted open reading frame (T20G5.5) in *C. elegans* cAMP-GEF (cel cAMP-GEF) (Fig. 3A). The cAMP binding domains of the cAMP-GEF family proteins form a distinct group within the cyclic nucleotide-binding protein superfamily and show the closest similarity to the B domains of PKA regulatory subunits (Fig. 3B). A PR(A/T)AT motif in the cAMP binding pocket is also conserved in the cAMP-GEF proteins (Fig. 3E). The first alanine of this motif confers cAMP (alanine) as opposed to cGMP (threonine) binding specificity. All of the cAMP-GEF family members have alanine at this position, and therefore bind cAMP rather than cGMP.

The GEF domains of the cAMP-GEFs show high homology to those of Ras-GEF family proteins, but form an independent cluster distinct from Ras GEFs such as mCdc25, hSos1, and rRas-GRF (Fig. 3, C and D). The three structurally conserved regions specific to Ras-GEF family proteins (SCR1, SCR2, and SCR3) are present in all of the cAMP-GEF proteins (Fig. 3D). Multiple sequence alignments and phylogenetic tree analyses were carried out with LASERGENE (DNASTAR Inc.). Abbreviations and GenBank accession numbers of the protein sequences used in this figure: hPKARI $\alpha$  (human cAMP-dependent protein kinase regulatory subunit type I-alpha): 125193, hPKARI $\beta$ : 1346362, hPKARII $\alpha$ : 125198, hPKARII $\beta$ : 400115, hPKGII $\alpha$  (human cGMP-dependent protein kinase type I-alpha): 1255602, hPKGII $\beta$ : 125379, hPKGII: 1906312, hCalDAG-GEFI: U71870, hCalDAG-GEFII: AF081195, C3G: 474982, hSos1 (human son-of-sevenless 1): 476780, mCdc25: 882120, rRas-GRF: 57665, BUD5: 171141.

In order to identify the small G protein substrate for cAMP-GEFI and II and the mode of cAMP regulation of GEF activity conferred by these proteins, the effects of cAMP-GEFI and cAMP-GEFII expression were analyzed in 293T cells on the ratio of GTP to GDP bound to different Ras family small G proteins in the presence or absence of forskolin and IBMX. Under basal conditions, in the absence of forskolin and IBMX, only Rap1 was activated significantly. In the presence of forskolin and IBMX, both cAMP-GEFI and II strongly and selectively activated Rap1A, but did not activate H-Ras, R-Ras or RalA. The effects of forskolin/IBMX treatment on cAMP-GEFI and II were dose-dependent with EC<sub>50</sub> values of 1.8  $\mu$ M and 0.3  $\mu$ M, respectively. Forskolin/IBMX treatment given alone had no effect.

- 52 -

A time-course analysis of the activation of Rap1A by forskolin/IBMX in cAMP-GEFI transfectants showed that the activation began within 10 sec, reached a maximum at 5 min, and continued for at least 60 min. Thus, cAMP-GEFI has a direct effect on Rap1A rather than secondary effects mediated by other Ras-superfamily GEFs. In addition, Sp-cAMPS, an analogue of cAMP, activated Rap1A at levels similar to those induced by forskolin/IBMX. Thus, cAMP has the capacity to activate the GEF domain of cAMP-GEFI.

Mutational analyses with cAMP-GEFI was performed to examine whether its cAMP-binding domain is required for the activation of Rap1A. In contrast to wild type cAMP-GEFI, a deletion mutant lacking a cAMP binding domain (pcDNA-rcAMP-GEFI:DcAMP(528) and (595)) did not activate Rap1A with or without forskolin/IBMX treatment. Mutants with a single amino acid substitution at the cAMP binding pocket (pcDNA-rcAMP-GEFI:R(279)K) responded minimally to forskolin/IBMX treatment. Thus, the cAMP binding domain of cAMP-GEFI is necessary for its cAMP-dependent activation of Rap1A.

To assess further the cAMP binding capacity of cAMP-GEFI, a cAMP agarose affinity bead binding assay was performed. *In vitro* translated, radiolabeled cAMP-GEFI showed selective binding to the beads that was competed by excess amounts of either cAMP or 8-Br-cAMP. cAMP-GEF protein can bind cAMP and that this binding can activate Rap1A.

cAMP-dependent activation of Rap1 has previously been ascribed to the phosphorylation of Rap1A by PKA, which raises its affinity to smgGDS, a GEF with broad substrate specificity. However, at least in the 293T cell assay system, an increase of GTP-bound Rap1A in response to increasing cAMP levels with forskolin or treatment with the cAMP analogue, Sp-cAMPS was not detected in the absence of cAMP-GEFs. In addition, even in the presence of H-89, a potent and selective inhibitor of PKA, cAMP-GEFI and II could still activate Rap1A. The activation of Rap1A induced by cAMP-GEFI and II is independent of the PKA pathway.

Intracellular cAMP has been shown to interact directly with ion channels, but the vast majority of cAMP-mediated effects in eukaryotes have been considered as sequels to cAMP binding by the regulatory subunits of the PKA tetramer. The diversity of physiological effects produced by cAMP have been attributed to the fact that, as a kinase, PKA has a large range of molecular targets. Reported herein are novel cAMP binding proteins that directly link the cAMP second messenger system to Ras superfamily signaling pathways and that appear selectively to target Rap.



cAMP can exert profound cell-type specific effects on cell growth and differentiation and that cAMP is capable of inhibiting or stimulating the Ras/mitogen-activated protein (MAP) kinase/Erk pathway. The inhibition can occur at the initial translocation step by which Ras activates Raf, whereas activation of Rap1 is thought to occur through phosphorylation by PKA.

5 Activation of Rap1 has been suggested to be part of a switch mechanism determining whether growth or differentiation occurs in response to nerve growth factor (NGF). cAMP-GEFs directly couple cAMP to Rap1, itself discovered as a negative regulator of Ras but suspected of having independent functions as well. Thus, different levels of cAMP-GEF expression confer cell-type specific regulation of Ras superfamily signaling systems.

10 The genes also exhibit developmentally regulated expression in the septum, medial thalamus and habenula, key structures in the limbic system variously linked to brain reward circuits, addiction and schizophrenia. Thus, cAMP-GEFs, in addition to PKA, underlie some of the neuronal functions of cAMP.

15 Example 3. Northern hybridization demonstrating the expression of CalDAG-GEFI and CalDAG-GEFII protein mRNAs in a variety of tissues.

Total cytoplasmic RNA was isolated from various human tissue samples including amygdala, cerebellum, corpus callosum, caudate nucleus, cortex, frontal lobe, hippocampus, kidney, liver, lung, medulla oblongata, occipital pole, putamen, spinal cord, substantia nigra, subthalamic nucleus, thalamus, and temporal lobe, obtained from surgical pathology using  
20 standard procedures such as CsCl purification. The RNA was then electrophoresed on a formaldehyde gel to permit size fractionation. The nitrocellulose membrane was prepared and the RNA was then transferred onto the membrane. <sup>32</sup>P-labeled cDNA probes were prepared and added to the membrane in order for hybridization between the probe the RNA to occur. After washing, the membrane was wrapped in plastic film and placed into imaging cassettes containing  
25 X-ray film. The autoradiographs were then allowed to develop for one to several days. Sizing was established by comparison to standard RNA markers. These northern blots demonstrated that the CalDAG-GEF genes are strongly expressed in the brain. Weaker hybridization was detectable elsewhere.

30 Example 4. Northern hybridization demonstrating the expression of cAMP-GEFI and cAMP-GEFII protein mRNAs in a variety of tissues.

- 54 -

Northern hybridization analysis was performed as in Example 3 to detect the expression of the cAMP-GEFI and cAMP-GEFII genes in a variety of human tissues. The tissues analyzed included adrenal gland, amygdala, bone marrow, cerebellum, corpus callosum, caudate nucleus, colon (mucosal lining), caudputamen, cortex, frontal lobe, hippocampus, habenula, heart, kidney, liver, lung, lymph node, medulla obongata, occipital pole, olfactory bulb, ovary, pons, pancreas, putamen, septum, small intestines, skeletal muscle, spinal cord, spleen, stomach, substantia nigra, subthalamic nucleus, testis, thalamus, temporal lobe, thymus, trachea, and thyroid.

A striking contrast in the expression patterns of human cAMP-GEFI and II was observed by Northern analysis. Human cAMP-GEFI is widely expressed, with highest levels appearing in kidney, spleen, thyroid, heart, and pancreas. Human cAMP-GEFII shows a remarkably selective enrichment in the brain and the adrenal glands. Both genes were developmentally regulated. The expression patterns of the two genes in the nervous system also differ, with cAMP-GEFI having a wider expression than cAMP-GEFII. These region-specific neuronal expression patterns were confirmed in *in situ* hybridization experiments. cAMP-GEFI mRNA is expressed broadly at low levels in the adult brain, but it is strongly and selectively expressed in parts of the neonatal brain, including the septum and the thalamus. By contrast, cAMP-GEFII is strongly expressed in the mature as well as the developing brain. Notable are the high levels of cAMP-GEFII mRNA in the cerebral cortex, the hippocampus (especially CA3 and dentate gyrus), the habenula and the cerebellum. Genes of the cAMP-GEF family have widespread influence on cAMP functions in bodily organs and also region-specific functions in the brain.

Example 5. Isolation of CalDAG-GEF or cAMP-GEF binding proteins by yeast two-hybrid system.

To identify proteins interacting with the CalDAG-GEF or cAMP-GEF proteins, a yeast expression plasmid vector (pAS2-1, Clontech) is generated by ligating an in-frame partial cDNA sequence encoding either residues of the CalDAG-GEF protein or residues of the cAMP-GEF protein into the EcoRI and BamHI sites of the vector. The resultant fusion protein contains the GAL4 DNA binding domain coupled in-frame either to residues of the CalDAG-GEF protein or to residues of the cAMP-GEF protein. These expression plasmids are co-transformed, along with purified plasmid DNA from the human brain cDNA:pACT library, into yeast using the protocols of the Clontech Matchmaker yeast-two-hybrid kit (Clontech). Yeast clones bearing human brain cDNAs which interact with the CalDAG-GEF or cAMP-GEF fragments are selected by HIS resistance and  $\beta$ gal<sup>+</sup> activation. The clones are further selected by

- 55 -

cyclohexamide sensitivity and the inserts of the human brain cDNAs are isolated by PCR and sequenced.

Although preferred embodiments of the invention have been described herein in detail, it will be understood by those skilled in the art that variations may be made thereto without  
5 departing from the spirit of the invention or the scope of the following claims.

- 56 -

CLAIMS

What is claimed is:

1. An isolated nucleic acid comprising a nucleotide sequence encoding a protein selected from the group consisting of a normal CalDAG-GEFI protein, a mutant CalDAG-GEFI protein, a normal CalDAG-GEFII protein, and a mutant CalDAG-GEFII protein.
2. An isolated nucleic acid comprising a nucleotide sequence encoding a protein selected from the group consisting of a normal cAMP-GEFI protein, a mutant cAMP-GEFI protein, a normal cAMP-GEFII protein, and a mutant cAMP-GEFII protein.
3. An isolated nucleic acid as in claim 1 wherein said nucleic acid encodes a normal CalDAG-GEF protein and wherein said nucleotide sequence is selected from the group consisting of
  - (a) a sequence encoding a protein comprising the human CalDAG-GEFI amino acid sequence of SEQ ID NO: 4;
  - (b) a sequence encoding a protein comprising the murine CalDAG-GEFI amino acid sequence of SEQ ID NO: 2;
  - (c) a sequence encoding a protein comprising the human CalDAG-GEFII amino acid sequence of SEQ ID NO: 8; and
  - (d) a sequence encoding a protein comprising the murine CalDAG-GEFII amino acid sequence of SEQ ID NO: 6; and
  - (e) a sequence encoding a normal CalDAG-GEF protein and capable of hybridizing to a sequence complementary to any sequence of (a) - (d) under stringent hybridization conditions.
4. An isolated nucleic acid as in claim 2 wherein said nucleic acid encodes a normal cAMP-GEF protein and wherein said nucleotide sequence is selected from the group consisting of
  - (a) a sequence encoding a protein comprising the human cAMP-GEFI amino acid sequence of SEQ ID NO: 12;
  - (b) a sequence encoding a protein comprising the alternatively spliced human cAMP-GEFI amino acid sequence of SEQ ID NO: 14;
  - (c) a sequence encoding a protein comprising the rat cAMP-GEFI amino acid sequence of SEQ ID NO: 10;
  - (d) a sequence encoding a protein comprising the human cAMP-GEFII amino acid sequence of SEQ ID NO: 18;

- 57 -

(e) a sequence encoding a protein comprising the rat cAMP-GEFII amino acid sequence of SEQ ID NO: 16; and

(f) a sequence encoding a normal cAMP-GEF protein and capable of hybridizing to a sequence complementary to any sequence of (a) - (e) under stringent hybridization conditions.

5. An isolated nucleic acid comprising a nucleotide sequence of at least 8 consecutive nucleotides selected from the group consisting SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, and a sequence complementary to any of these sequences.

6. An isolated nucleic acid comprising a nucleotide sequence of at least 10 consecutive nucleotides selected from the group consisting SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, and a sequence complementary to any of these sequences.

7. An isolated nucleic acid comprising a nucleotide sequence of at least 15 consecutive nucleotides selected from the group consisting SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, and a sequence complementary to any of these sequences.

8. An isolated nucleic acid comprising a nucleotide sequence encoding at least one functional domain of a CalDAG-GEF protein selected from the group consisting of a normal CalDAG-GEFI protein, a mutant CalDAG-GEFI protein, a normal CalDAG-GEFII protein, and a mutant CalDAG-GEFII protein.

9. An isolated nucleic acid comprising a nucleotide sequence encoding at least one functional domain of a cAMP-GEF protein selected from the group consisting of a normal cAMP-GEFI protein, a normal cAMP-GEFII protein, a mutant cAMP-GEFI protein, and a mutant cAMP-GEFII protein.

10. An isolated nucleic acid comprising a nucleotide sequence encoding an antigenic determinant of a CalDAG-GEF protein selected from the group consisting of a normal CalDAG-GEFI protein, a normal CalDAG-GEFII protein, a mutant CalDAG-GEFI protein, and a mutant CalDAG-GEFII protein.

11. An isolated nucleic acid comprising a nucleotide sequence encoding an antigenic determinant of a cAMP-GEF protein selected from the group consisting of a normal cAMP-GEFI protein, a normal cAMP-GEFII protein, a mutant cAMP-GEFI protein, and a mutant cAMP-GEFII protein.

- 58 -

1 12. A method for identifying an allelic variant or heterospecific homologue of a human  
2 CalDAG-GEF gene comprising:

3 choosing a nucleic acid probe or primer capable of hybridizing to a human CalDAG-  
4 GEF gene sequence under stringent hybridization conditions;

5 mixing said probe or primer with a sample of nucleic acids which may contain a  
6 nucleic acid corresponding to said variant or homologue; and

7 detecting hybridization of said probe or primer to said nucleic acid corresponding to  
8 said variant or homologue.

1 13. A method as in claim 12 wherein said human CalDAG-GEF gene sequence is  
2 selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 7.

1 14. A method as in claim 12 wherein said sample comprises a sample of nucleic acids  
2 selected from the group consisting of human genomic DNA, human mRNA, and human cDNA.

1 15. A method as in claim 12 wherein said sample comprises a sample of nucleic acids  
2 selected from the group consisting of mammalian genomic DNA, mammalian mRNA, and  
3 mammalian cDNA.

1 16. A method as in claim 12 wherein said sample comprises a sample of nucleic acids  
2 selected from the group consisting of invertebrate genomic DNA, invertebrate mRNA, and  
3 invertebrate cDNA.

1 17. A method as in claim 12 further comprising the step of isolating said nucleic acid  
2 corresponding to said variant or homologue.

1 18. A method as in claim 12 wherein said nucleic acid is identified by hybridization.

1 19. A method as in claim 12 wherein said nucleic acid is identified by PCR amplification.

1 20. A method for identifying allelic variants or heterospecific homologues of a human  
2 cAMP-GEF gene comprising:

3 choosing a nucleic acid probe or primer capable of hybridizing to a human cAMP-  
4 GEF gene sequence under stringent hybridization conditions;

5 mixing said probe or primer with a sample of nucleic acids which may contain a  
6 nucleic acid corresponding to said variant or homologue; and

7 detecting hybridization of said probe or primer to said nucleic acid corresponding to  
8 said variant or homologue.

1 21. A method as in claim 12 wherein said human cAMP-GEF gene sequence is selected  
2 from the group consisting of SEQ ID NO: 11, SEQ ID NO: 13, and SEQ ID NO: 17.

- 59 -

- 1 22. A method as in claim 20 wherein said sample comprises a sample of nucleic acids  
2 selected from the group consisting of human genomic DNA, human mRNA, and human cDNA.
- 1 23. A method as in claim 20 wherein said sample comprises a sample of nucleic acids  
2 selected from the group consisting of mammalian genomic DNA, mammalian mRNA, and  
3 mammalian cDNA.
- 1 24. A method as in claim 20 wherein said sample comprises a sample of nucleic acids  
2 selected from the group consisting of invertebrate genomic DNA, invertebrate mRNA, and  
3 invertebrate cDNA.
- 1 25. A method as in claim 20 further comprising the step of isolating said nucleic acid  
2 corresponding to said variant or homologue.
- 1 26. A method as in claim 20 wherein said nucleic acid is identified by hybridization.
- 1 27. A method as in claim 20 wherein said nucleic acid is identified by PCR amplification.
- 1 28. A method for identifying an allelic variant or heterospecific homologue of a human  
2 CalDAG-GEF gene comprising:  
3 choosing an antibody capable of selectively binding to a human CalDAG-GEF  
4 protein;  
5 mixing said antibody with a sample of proteins which may contain a protein  
6 corresponding to said variant or homologue; and  
7 detecting binding of said antibody to said protein corresponding to said variant or  
8 homologue.
- 1 29. A method as in claim 28 wherein said sample comprises a sample of proteins selected  
2 from the group consisting of human proteins, human fusion proteins, and proteolytic fragments  
3 thereof.
- 1 30. A method as in claim 28 wherein said sample comprises a sample of nucleic acids  
2 selected from the group consisting of mammalian proteins, mammalian fusion proteins, and  
3 proteolytic fragments thereof.
- 1 31. A method as in claim 28 wherein said sample comprises a sample of nucleic acids  
2 selected from the group consisting of invertebrate proteins, invertebrate fusion proteins, and  
3 proteolytic fragments thereof.
- 1 32. A method as in claim 28 further comprising the step of substantially purifying said  
2 protein corresponding to said variant or homologue.

- 60 -

1 33. A method for identifying an allelic variant or heterospecific homologue of a human  
2 cAMP-GEF gene comprising:

3 choosing an antibody capable of selectively binding to a human cAMP-GEF protein;  
4 mixing said antibody with a sample of proteins which may contain a protein  
5 corresponding to said variant or homologue; and  
6 detecting binding of said antibody to said protein corresponding to said variant or  
7 homologue.

1 34. A method as in claim 33 wherein said sample comprises a sample of proteins selected  
2 from the group consisting of human proteins, human fusion proteins, and proteolytic fragments  
3 thereof.

1 35. A method as in claim 33 wherein said sample comprises a sample of proteins selected  
2 from the group consisting of mammalian proteins, mammalian fusion proteins, and proteolytic  
3 fragments thereof.

1 36. A method as in claim 33 wherein said sample comprises a sample of proteins selected  
2 from the group consisting of invertebrate proteins, invertebrate fusion proteins, and proteolytic  
3 fragments thereof.

1 37. A method as in claim 33 further comprising the step of substantially purifying said  
2 protein corresponding to said variant or homologue.

1 38. An isolated nucleic acid comprising an allelic variant or a heterospecific homologue  
2 of a gene selected from the group consisting of a human CalDAG-GEF gene, and a human  
3 cAMP-GEF gene.

1 39. An isolated nucleic acid encoding an allelic variant or heterospecific homologue of a  
2 protein selected from the group consisting of a human CalDAG-GEF protein, and a human  
3 cAMP-GEF protein.

1 40. An isolated nucleic acid comprising a recombinant vector including a nucleotide  
2 sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5,  
3 SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO:  
4 17, and a sequence complementary to any of these sequences.

1 41. An isolated nucleic acid as in claim 40 wherein said vector is an expression vector  
2 and said nucleotide sequence is operably joined to a regulatory region.

1 42. An isolated nucleic acid as in claim 41 wherein said expression vector may express  
2 said nucleotide sequence in mammalian cells.



- 61 -

- 1 43. An isolated nucleic acid as in claim 42 wherein said cells are selected from the group  
2 consisting of fibroblast, liver, kidney, spleen, bone marrow, and neurological cells.
- 1 44. An isolated nucleic acid as in claim 42 wherein said vector is selected from the group  
2 consisting of vaccinia virus, adenovirus, retrovirus, neurotropic viruses, and Herpes simplex.
- 1 45. An isolated nucleic acid as in claim 41 wherein said expression vector encodes at  
2 least a functional domain of a protein selected from the group consisting of normal CalDAG-  
3 GEFI, a normal CalDAG-GEFII, a mutant CalDAG-GEFI, a mutant CalDAG-GEFII, a normal  
4 cAMP-GEFI, a normal cAMP-GEFII, a mutant cAMP-GEFI, and a mutant cAMP-GEFII.
- 1 46. An isolated nucleic acid as in claim 41 wherein said vector further comprises  
2 sequences encoding an exogenous protein operably joined to said nucleotide sequence and  
3 whereby said vector encodes a fusion protein.
- 1 47. An isolated nucleic acid as in claim 46 wherein said exogenous protein is selected  
2 from the group consisting of lacZ, trpE, maltose-binding protein, poly-His tags, and glutathione-  
3 S-transferase.
- 1 48. An isolated nucleic acid comprising a recombinant expression vector including  
2 nucleotide sequences corresponding to an endogenous regulatory region of a gene selected from  
3 the group consisting of a CalDAG-GEF gene, and a cAMP-GEF gene.
- 1 49. An isolated nucleic acid as in claim 48 wherein said endogenous regulatory region is  
2 operably joined to a marker gene.
- 1 50. A host cell transformed with an expression vector of any one of claims 41-49, or a  
2 descendant thereof.
- 1 51. A host cell as in claim 50 wherein said host cell is selected from the group consisting  
2 of bacterial cells and yeast cells.
- 1 52. A host cell as in claim 50 wherein said host cell is selected from the group consisting  
2 of fetal cells, embryonic stem cells, zygotes, gametes, and germ line cells.
- 1 53. A host cell as in claim 50 wherein said cell is selected from the group consisting of  
2 fibroblast, liver, kidney, spleen, bone marrow and neurological cells.
- 1 54. A host cell as in claim 50 wherein said cell is an invertebrate cell.
- 1 55. A non-human animal model for cancer, wherein a genome of said animal, or an  
2 ancestor thereof, has been modified by at least one recombinant construct, and wherein said  
3 recombinant construct has introduced a modification selected from the group consisting of

- 62 -

(a) insertion of nucleotide sequences encoding at least a functional domain of a heterospecific normal CalDAG-GEF gene;

(b) insertion of nucleotide sequences encoding at least a functional domain of a heterospecific mutant CalDAG-GEF gene;

(c) insertion of nucleotide sequences encoding at least a functional domain of a conspecific homologue of a heterospecific mutant CalDAG-GEF gene;

(d) inactivation of an endogenous CalDAG-GEF gene;

(e) insertion of nucleotide sequences encoding at least a functional domain of a heterospecific normal cAMP-GEF gene;

(f) insertion of nucleotide sequences encoding at least a functional domain of a heterospecific mutant cAMP-GEF gene;

(g) insertion of nucleotide sequences encoding at least a functional domain of a conspecific homologue of a heterospecific mutant cAMP-GEF gene; and

(h) inactivation of an endogenous cAMP-GEF gene.

56. A non-human animal model as in claim 55 wherein said cancer is related to the Ras-pathway.

57. A non human animal model as in claim 56 wherein said cancer is selected from the group consisting of lung cancer, pancreatic cancer, breast cancer, colorectal cancer, and myeloid leukemia.

58. An animal model as in claim 55 wherein said modification is an insertion of a nucleotide sequence encoding at least a functional domain of a protein selected from the group consisting of a normal human CalDAG-GEF, and a normal cAMP-GEF gene.

59. An animal model as in claim 55 wherein said modification is an insertion of a nucleotide sequence encoding at least a functional domain of a protein selected from the group consisting of a mutant human CalDAG-GEF, and a mutant human cAMP-GEF gene.

60. An animal as in claim 55 wherein said animal is selected from the group consisting of rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates.

61. An animal as in claim 55 wherein said animal is an invertebrate.

62. A method for producing at least a functional domain of a protein selected from the group consisting of a CalDAG-GEF protein, and a cAMP-GEF protein, said method comprising culturing a host cell of any of claims 50-54 under suitable conditions to produce said protein by expressing said nucleic acid.

- 63 -

63. A substantially pure preparation of a protein selected from the group consisting of a normal CalDAG-GEF protein, a mutant CalDAG-GEF protein, a normal cAMP-GEF protein, and a mutant cAMP-GEF protein.

64. A substantially pure preparation as in claim 63 wherein said protein comprises a normal protein selected from the group consisting of

(a) a protein comprising the amino acid sequence of SEQ ID NO: 2;

(b) a protein comprising the amino acid sequence of SEQ ID NO: 4;

(c) a protein comprising the amino acid sequence of SEQ ID NO: 6;

(d) a protein comprising the amino acid sequence of SEQ ID NO: 8;

(e) a protein comprising the amino acid sequence of SEQ ID NO: 10;

(f) a protein comprising the amino acid sequence of SEQ ID NO: 12;

(g) a protein comprising the amino acid sequence of SEQ ID NO: 14;

(h) a protein comprising the amino acid sequence of SEQ ID NO: 16; and

(i) a protein comprising the amino acid sequence of SEQ ID NO: 18.

65. A substantially pure preparation of a polypeptide comprising an amino acid sequence of at least 4 consecutive amino acid residues selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, and SEQ ID NO: 18.

66. A substantially pure preparation of a polypeptide comprising an amino acid sequence of at least 10 consecutive amino acid residues selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, and SEQ ID NO: 18.

67. A substantially pure preparation of a polypeptide comprising an amino acid sequence of at least 15 consecutive amino acid residues selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, and SEQ ID NO: 18.

68. A substantially pure preparation of a polypeptide comprising at least one functional domain of a protein selected from the group consisting of a normal CalDAG-GEF protein, a mutant CalDAG-GEF protein, a normal cAMP-GEF protein, and a mutant cAMP-GEF protein.

69. A substantially pure preparation of a polypeptide comprising an antigenic determinant of a protein selected from the group consisting of a normal CalDAG-GEF protein, a mutant CalDAG-GEF protein, a normal cAMP-GEF protein, and a mutant cAMP-GEF protein.

- 64 -

1 70. A method of producing antibodies which selectively bind to a CalDAG-GEF protein  
2 comprising the steps of

3 administering an immunogenically effective amount of a CalDAG-GEF immunogen  
4 to an animal;

5 allowing said animal to produce antibodies to said immunogen; and

6 obtaining said antibodies from said animal or from a cell culture derived therefrom.

1 71. A method of producing antibodies which selectively bind to a cAMP-GEF protein  
2 comprising the steps of

3 administering an immunogenically effective amount of a cAMP-GEF immunogen to  
4 an animal;

5 allowing said animal to produce antibodies to said immunogen; and

6 obtaining said antibodies from said animal or from a cell culture derived therefrom.

1 72. A substantially pure preparation of an antibody which selectively binds to an  
2 antigenic determinant of a protein selected from the group consisting of a normal CalDAG-GEF  
3 protein, a mutant CalDAG-GEF protein, a normal cAMP-GEF protein, and a mutant cAMP-GEF  
4 protein.

1 73. A substantially pure preparation of an antibody as in claim 72 wherein said antibody  
2 selectively binds to an antigenic determinant of a mutant CalDAG-GEF and fails to bind to a  
3 normal CalDAG-GEF protein.

1 74. A substantially pure preparation of an antibody as in claim 72 wherein said antibody  
2 selectively binds to an antigenic determinant of a mutant cAMP-GEF and fails to bind to a  
3 normal cAMP-GEF protein.

1 75. A cell line producing an antibody of any one of claims 72-74.

1 76. A method for identifying compounds which can modulate the expression of a  
2 CalDAG-GEF gene comprising:

3 contacting a cell with a test candidate wherein said cell includes a regulatory region of  
4 a CalDAG-GEF gene operably joined to a coding region; and

5 detecting a change in expression of said coding region.

1 77. A method for identifying compounds which can modulate the expression of a cAMP-  
2 GEF gene comprising:

3 contacting a cell with a test candidate wherein said cell includes a regulatory region of  
4 a cAMP-GEF gene operably joined to a coding region; and

- 65 -

- 5 detecting a change in expression of said coding region.
- 1 78. A method as in claim 76 or 77 wherein said change comprises a change in a level of  
2 an mRNA transcript encoded by said coding region.
- 1 79. A method as in claim 78 wherein said change comprises a change in a level of a  
2 protein encoded by said coding region.
- 1 80. A method as in claim 78 wherein said change is a result of an activity of a protein  
2 encoded by said coding region.
- 1 81. A method as in claim 78 wherein said coding region encodes a marker protein  
2 selected from the group consisting of  $\beta$ -galactosidase, alkaline phosphatase, green fluorescent  
3 protein, and luciferase.
- 1 82. A method for identifying compounds which can selectively bind to a CalDAG-GEF  
2 protein comprising the steps of  
3 providing a preparation including at least one CalDAG-GEF component;  
4 contacting said preparation with a sample including at least one candidate compound;  
5 and  
6 detecting binding of said CalDAG-GEF component to said candidate compound.
- 1 83. A method for identifying compounds which can selectively bind to a cAMP-GEF  
2 protein comprising the steps of  
3 providing a preparation including at least one cAMP-GEF component;  
4 contacting said preparation with a sample including at least one candidate compound;  
5 and  
6 detecting binding of said cAMP-GEF component to said candidate compound.
- 1 84. The method in claim 82 wherein said binding to said CalDAG-GEF component is  
2 detected by an assay selected from the group consisting of: affinity chromatography, co-  
3 immunoprecipitation, a Biomolecular Interaction Assay, and a yeast two-hybrid system.
- 1 85. The method in claim 83 wherein said binding to said cAMP-GEF component is  
2 detected by an assay selected from the group consisting of: affinity chromatography, co-  
3 immunoprecipitation, a Biomolecular Interaction Assay, and a yeast two-hybrid system.
- 1 86. A method of identifying compounds which can modulate activity of a CalDAG-GEF  
2 comprising the steps of  
3 providing a cell expressing a normal or mutant CalDAG-GEF gene;  
4 contacting said cell with at least one candidate compound; and

- 66 -

5 detecting a change in a marker of said activity.

1 87. A method of identifying compounds which can modulate activity of a cAMP-GEF  
2 comprising the steps of

3 providing a cell expressing a normal or mutant cAMP-GEF gene;

4 contacting said cell with at least one candidate compound; and

5 detecting a change in a marker of said activity.

1 88. A method as in claim 86 wherein measurement of said marker indicates a difference  
2 between cells bearing an expressed mutant CalDAG-GEF gene and otherwise identical cells free  
3 of an expressed mutant CalDAG-GEF gene.

1 89. A method as in claim 86 wherein said change comprises a change in a non-specific  
2 marker of cell physiology selected from the group consisting of pH, intracellular calcium, cyclic  
3 AMP levels, GTP/GDP ratios, phosphatidylinositol activity, and protein phosphorylation.

1 90. A method as in claim 86 wherein said change comprises a change in expression of  
2 said CalDAG-GEF.

1 91. A method as in claim 86 wherein said change comprises a change in occurrence or  
2 rate of apoptosis or cell death.

1 92. A method as in claim 86 wherein said cell is a cell cultured *in vitro*.

1 93. A method as in claim 92 wherein said cell is a transformed host cell of any one of  
2 claims 50-54.

1 94. A method as in claim 92 wherein said cell is explanted from a host bearing at least  
2 one mutant CalDAG-GEF gene.

1 95. A method as in claim 92 wherein said cell is explanted from a transgenic animal of  
2 any one of claims 55-61.

1 96. A method as in claim 86 wherein said cell is a cell in a live animal.

1 97. A method as in claim 96 wherein said cell is a cell of a transgenic animal of any one  
2 of claims 55-61.

1 98. A method as in claim 86 wherein said cell is in a human subject in a clinical trial.

1 99. A method as in claim 87 wherein measurement of said marker indicates a difference  
2 between cells bearing an expressed mutant cAMP-GEF gene and otherwise identical cells free of  
3 an expressed mutant cAMP-GEF gene.

- 67 -

1 100. A method as in claim 87 wherein said change comprises a change in a non-specific  
2 marker of cell physiology selected from the group consisting of pH, intracellular calcium, cyclic  
3 AMP levels, GTP/GDP ratios, phosphatidylinositol activity, and protein phosphorylation.

1 101. A method as in claim 87 wherein said change comprises a change in expression of  
2 said cAMP-GEF.

1 102. A method as in claim 87 wherein said change comprises a change in occurrence or  
2 rate of apoptosis or cell death.

1 103. A method as in claim 87 wherein said cell is a cell cultured *in vitro*.

1 104. A method as in claim 103 wherein said cell is a transformed host cell of any one of  
2 claims 50-54.

1 105. A method as in claim 103 wherein said cell is explanted from a host bearing at least  
2 one mutant cAMP-GEF gene.

1 106. A method as in claim 103 wherein said cell is explanted from a transgenic animal of  
2 any one of claims 55-61.

1 107. A method as in claim 87 wherein said cell is a cell in a live animal.

1 108. A method as in claim 107 wherein said cell is a cell of a transgenic animal of any one  
2 of claims 55-61.

1 109. A method as in claim 87 wherein said cell is in a human subject in a clinical trial.

1 110. A diagnostic method for determining if a subject bears a mutant CalDAG-GEF gene  
2 comprising the steps of

3 providing a biological sample of said subject; and

4 detecting in said sample a mutant CalDAG-GEF nucleic acid, a mutant CalDAG-GEF  
5 protein, or a mutant CalDAG-GEF activity.

1 111. A method as in claim 111, wherein a mutant CalDAG-GEF nucleic acid is detected  
2 by an assay selected from the group consisting of direct nucleotide sequencing, probe specific  
3 hybridization, restriction enzyme digest and mapping, PCR mapping, ligase-mediated PCR  
4 detection, RNase protection, electrophoretic mobility shift detection, and chemical mismatch  
5 cleavage.

1 112. A method as in claim 110, wherein a mutant CalDAG-GEF protein is detected by an  
2 assay selected from the group consisting of an immunoassay, a protease assay, and an  
3 electrophoretic mobility assay.

- 68 -

1 113. A diagnostic method for determining if a subject bears a mutant cAMP-GEF gene  
2 comprising the steps of

3 providing a biological sample of said subject; and

4 detecting in said sample a mutant cAMP-GEF nucleic acid, a mutant cAMP-GEF  
5 protein, or a mutant cAMP-GEF activity.

1 114. A method as in claim 113, wherein a mutant cAMP-GEF nucleic acid is detected by  
2 an assay selected from the group consisting of direct nucleotide sequencing, probe specific  
3 hybridization, restriction enzyme digest and mapping, PCR mapping, ligase-mediated PCR  
4 detection, RNase protection, electrophoretic mobility shift detection, and chemical mismatch  
5 cleavage.

1 115. A method as in claim 113, wherein a mutant cAMP-GEF protein is detected by an  
2 assay selected from the group consisting of an immunoassay, a protease assay, and an  
3 electrophoretic mobility assay.

1 116. A pharmaceutical preparation comprising a substantially pure CalDAG-GEF protein  
2 and a pharmaceutically acceptable carrier.

1 117. A pharmaceutical preparation comprising a substantially pure cAMP-GEF protein and  
2 a pharmaceutically acceptable carrier.

1 118. A pharmaceutical preparation comprising an expression vector operably encoding a  
2 protein selected from the group consisting of a CalDAG-GEF protein, and a cAMP-GEF protein,  
3 wherein said expression vector may express said CalDAG-GEF protein or said cAMP-GEF  
4 protein in a human subject, and a pharmaceutically acceptable carrier.

1 119. A pharmaceutical preparation comprising an expression vector operably encoding a  
2 CalDAG-GEF antisense sequence, wherein said expression vector may express said CalDAG-  
3 GEF antisense sequence in a human subject, and a pharmaceutically acceptable carrier.

1 120. A pharmaceutical preparation comprising an expression vector operably encoding a  
2 cAMP-GEF antisense sequence, wherein said expression vector may express said cAMP-GEF  
3 antisense sequence in a human subject, and a pharmaceutically acceptable carrier.

1 121. A pharmaceutical preparation comprising a substantially pure antibody, and a  
2 pharmaceutically acceptable carrier,

3 wherein said antibody selectively binds to a mutant protein selected from the group  
4 consisting of a mutant CalDAG-GEF protein, and a mutant cAMP-GEF protein.



- 69 -

1 122. A pharmaceutical preparation as in claim 121 wherein said preparation is essentially  
2 free of an antibody which selectively binds a normal CalDAG-GEF protein.

1 123. A pharmaceutical preparation as in claim 121 wherein said preparation is essentially  
2 free of an antibody which selectively binds a normal cAMP-GEF protein.

1 124. A pharmaceutical preparation comprising a substantially pure preparation of an  
2 antigenic determinant of a mutant CalDAG-GEF protein or a mutant cAMP-GEF protein.

1 125. A pharmaceutical preparation as in claim 124 wherein said preparation is essentially  
2 free of an antigenic determinant of a normal CalDAG-GEF protein.

1 126. A pharmaceutical preparation as in claim 124 wherein said preparation is essentially  
2 free of an antigenic determinant of a normal cAMP-GEF protein.

1 127. A method of treatment for a patient bearing a mutant CalDAG-GEF gene comprising  
2 the step of administering to said patient a therapeutically effective amount of the pharmaceutical  
3 preparation of claim 116.

1 128. A method for identifying compounds according to claim 82, wherein the CalDAG-  
2 GEF component is a CalDAG-GEF domain selected from the group consisting of SCR1, SCR2,  
3 SCR3, DAG-binding and an EF hand domain.

1 129. A substantially pure preparation of a polypeptide comprising a domain selected from  
2 the group consisting of a CalDAG-GEF SCR1 domain, a CalDAG-GEF SCR2 domain,  
3 CalDAG-GEF SCR3 domain, CalDAG-GEF DAG-binding domain, CalDAG-GEF EF hand  
4 domain.

1 130. A substantially pure preparation of a polypeptide comprising a domain selected from  
2 the group consisting of a cAMP-GEF SCR1 domain, a cAMP-GEF SCR2 domain, cAMP-GEF  
3 SCR3 domain, cAMP-GEF cAMP-binding domain.

1/12

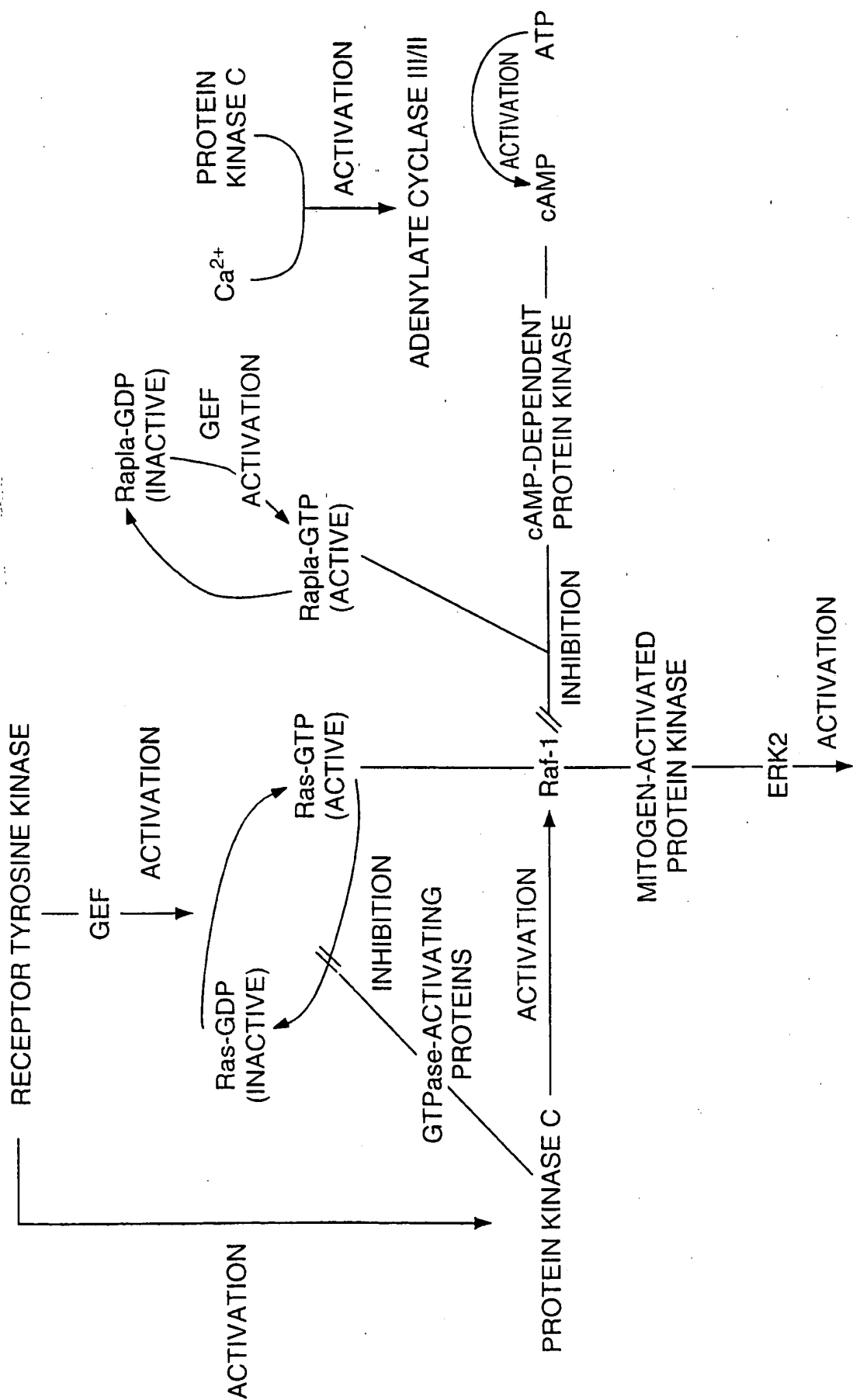


FIG. 1

MAPK-ACTIVATED PROTEIN KINASE 2, ETC.

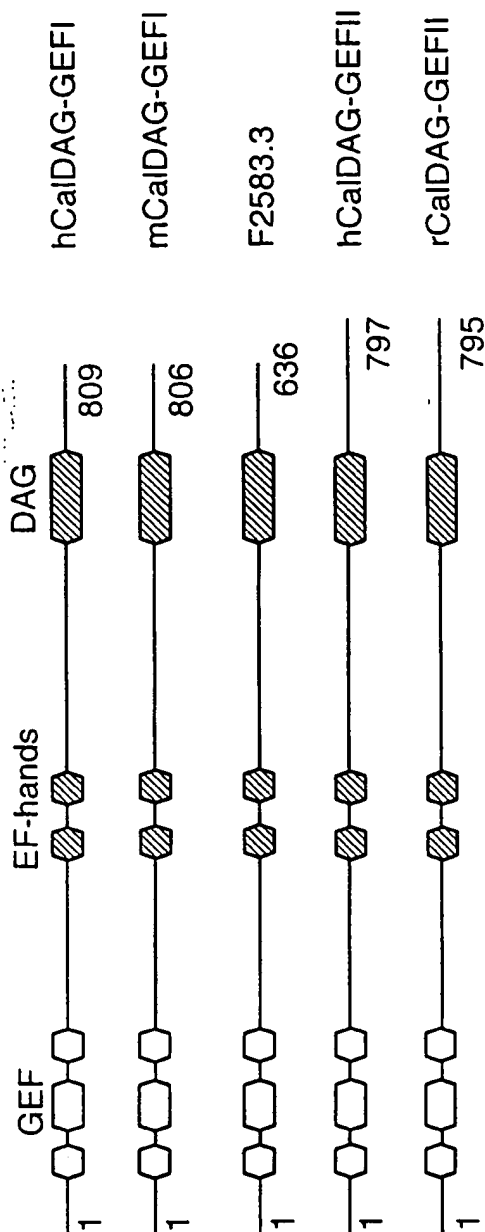


FIG. 2A

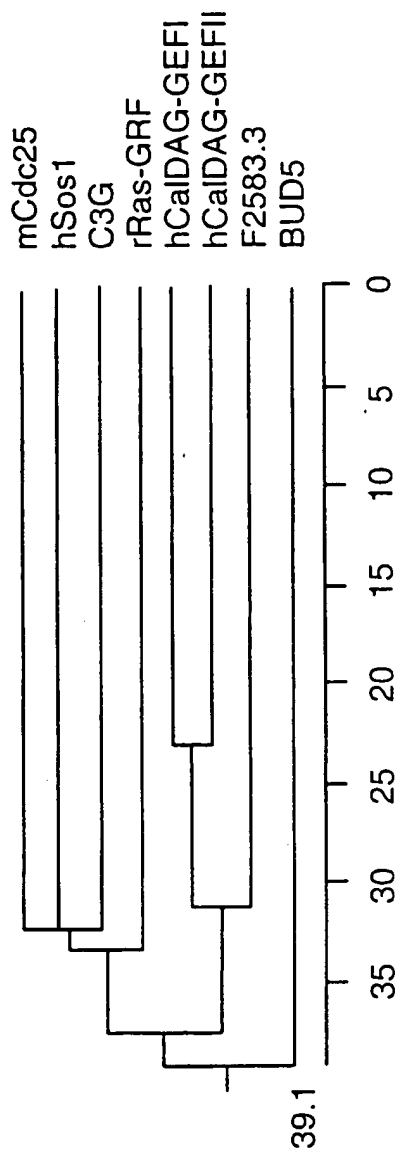


FIG. 2B

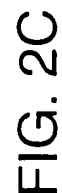


FIG. 2D-1	FIG. 2D-2
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FIG. 2D

hCaIDAG-GEFI	MAGTLDLDKGC	TVEEELLRGC	IEAFDDSGK	VRDPQLV	RMFLMMHP	WYIPSS
mCaIDAG-GEFI	MA[S]TLDLDKGC	TVEEELLRGC	IEAFDDSGK	VRDPQLV	RMFLMMHP	WYIPSS
hCaIDAG-GEFI	KE L K A L L D Q E G N R R H S S L I D I D S V P T Y K W K R Q V T Q R N P V G Q K K R K M S L L F					
mCaIDAG-GEFI	KE L K A L L D Q E G N R R H S S L I D I [E] S V P T Y K W K R Q V T Q R N P V [E] Q K K R K M S L L F					
hCaIDAG-GEFI	N S V S Q W V Q L M I L S K P T A P Q R A L V I T H F V H V A E K L L Q L Q N F N T L M A V V G G L					
mCaIDAG-GEFI	N S V S Q W V Q L M I L S K P T A [T] Q R A L V I T H F V H V A E K L L Q L Q N F N T L M A V V G G L					
hCaIDAG-GEFI	F P I L G V H L K D L V A L Q L A L P D W L D P A R T R L N G A K M K Q L F S I L E E L A M V T S L					
mCaIDAG-GEFI	F P I L G V H L K D L V A L Q L A L P D W L D P [G] R T R L N G A K M [R] Q L F S I L E E L A M V T S L					
hCaIDAG-GEFI	P P R P P V L E E W Y S A A K P K L D Q A L V V E H I E K M V E S V F R N F D V D G D G H I S Q E E					
mCaIDAG-GEFI	P P R P P V L E E W Y S [Y] A K P K L D Q A L V [A] E H I E K M V E S V F R N F D V D G D G H I S Q E E					
hCaIDAG-GEFI	F Q E S N S L R P V A C R H C K A L I L G I Y K Q G L K C R A C G V N C H K Q C K D R R L S L E C R R					
mCaIDAG-GEFI	F Q E S N S L R P V A C R H C K A L I L G I Y K Q G L K C R A C G V N C H K Q C K [E] R L S L E C R R					
hCaIDAG-GEFI	EDGVFDIHL					
mCaIDAG-GEFI	EDGVFDIHL					

FIG. 2D-1

Q L A A K L L H I Y Q Q S R K D N S N S L Q Y K T C H L V R Y W I S A F P A E F D L N P E L A E Q I	100
Q L A S K L L H F Y Q Q S R K D N S N S L Q Y K T C H L V R Y W V S A F P A E F D L N P E L A E P I	100
D H L E P M E L A E H L T Y L E Y R S F C K I L F Q D D Y H S F V T H G C T V D N P V L E R F I S L F	200
D H L E P M E L A E H L T Y L E Y R S F C K I L F Q D D Y H S F V T H G C T V D N P V L E R F I S L F	200
S H S S I S R L K E T H S H V S P E T I K L W E G L T E L V T A T G N Y G N Y R R R L A A C V G F R	300
S H S S I S R L K E T H S H V S P D T I K L W E G L T E L V T A T G N Y S N Y R R R L A A C V G F R	300
R P P V Q A N P D L L S L L T V S L D Q Y Q T E D E L Y Q L S L Q R E P R S K S S P T S P T S C T P	400
R P P V Q A K P S L L S L L T V S L D Q Y Q T E D E L Y Q L S L Q K E P R S K S S P T S P T S C T P	400
F Q I I R G N F P Y L S A F G D L O Q N Q D G C I S R E E M V S Y F L R S S S V L G G R M G F V H N	500
F Q I I R G N F P Y L S A F G D L O Q N Q D G C I S R E E M I S Y F L R S S S V L G G R M G F V H N	500
R A Q S V S L E G S A P S P S P M H S H H H R A F S F S L P R P G R R G S R P P E I R E E E V Q T V	600
R A Q S V S L E G S A P S P S P T H T H H H R A F S F S L P R P G R R G S R P P E I R E E E V Q S V	599
	609
	608

FIG. 2D-2

6/12

F2583.3	A	V	F	K	H	Y	D	H	D	R	D	G	F	I	S	Q	E	E	F	Q	473
hCaIDAG-GEFI	S	V	F	R	N	F	D	V	D	G	D	G	H	I	S	Q	E	E	F	Q	452
hCaIDAG-GEFII	S	V	F	K	N	Y	D	H	D	Q	D	G	Y	I	S	Q	E	E	F	E	447
hCalmodulin	E	A	F	S	L	F	D	K	D	G	D	G	T	I	T	K	F	L	G		34
hCalbindin D28K	L	M	L	K	L	F	D	S	N	N	D	G	K	L	E	L	T	E	M	A	168
hCalcineurin B	F	A	F	R	I	Y	D	M	D	K	D	G	Y	I	S	N	G	E	L	F	113
hParvalbumin $\alpha$	K	V	F	H	M	L	D	K	D	K	S	G	F	I	E	E	D	E	L	G	65
hTroponin C	E	C	F	R	I	F	D	R	K	A	D	G	Y	I	D	P	E	E	L	A	117

FIG. 2E

F2583.3	H	N	F	H	E	T	T	F	L	T	P	T	T	C	N	H	C	N	K	L	L	W	G	I	L	R	Q	G	F	K	C	K	D	C	G	L	A	V	H	S	C	C	K	S	N	A	V	A	E	C	570
hCaIDAG-GEFI	H	N	F	Q	E	S	N	S	L	R	P	V	A	C	R	H	C	K	A	L	I	L	G	I	Y	K	Q	G	L	K	C	R	A	C	G	V	N	C	H	K	Q	C	K	D	R	L	S	V	E	C	548
hCaIDAG-GEFII	H	N	F	Q	E	T	T	Y	L	K	P	T	F	C	D	N	C	A	G	F	L	W	G	V	I	K	Q	G	Y	R	C	C	D	C	C	M	N	C	H	K	Q	C	K	D	L	V	F	E	C	542	
hPKC $\alpha$	H	K	F	I	A	R	F	F	K	Q	P	T	F	C	S	H	C	T	D	F	I	W	G	F	G	K	Q	G	F	Q	C	Q	V	C	C	F	V	V	H	K	R	C	H	E	F	V	T	F	S	C	86
hPKC $\beta$ 1	H	K	F	T	A	R	F	F	K	Q	P	T	F	C	S	H	C	T	D	F	I	W	G	F	G	K	Q	G	F	Q	C	Q	V	C	C	F	V	V	H	K	R	C	H	E	F	V	T	F	S	C	86
hPKC $\gamma$	H	K	F	T	A	R	F	F	K	Q	P	T	F	C	S	H	C	T	D	F	I	W	G	I	G	K	Q	G	L	Q	C	Q	V	C	S	F	V	V	H	R	C	H	E	F	V	T	F	E	C	85	

FIG. 2F

7/12

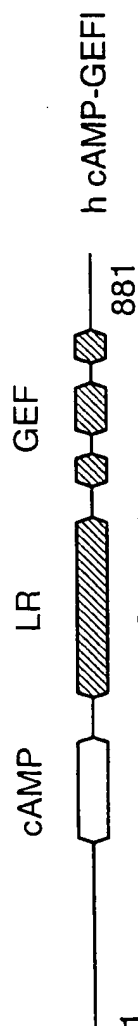


FIG. 3A

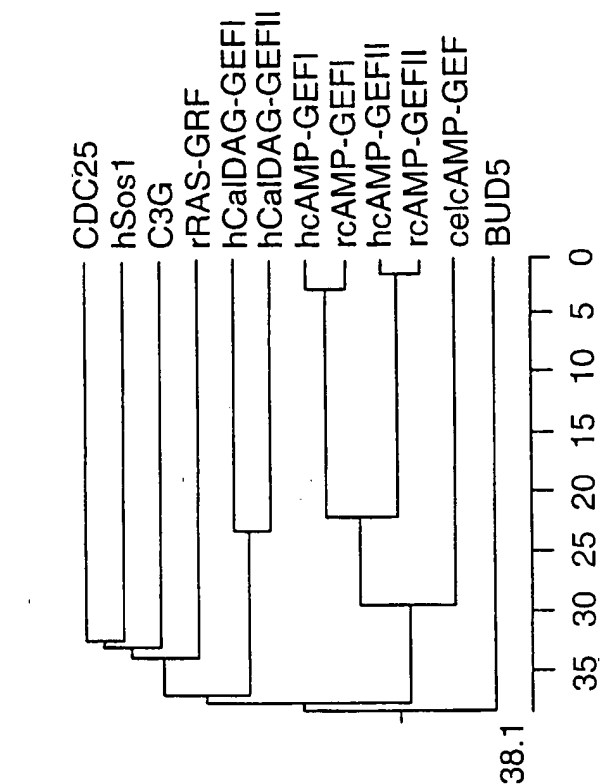


FIG. 3C

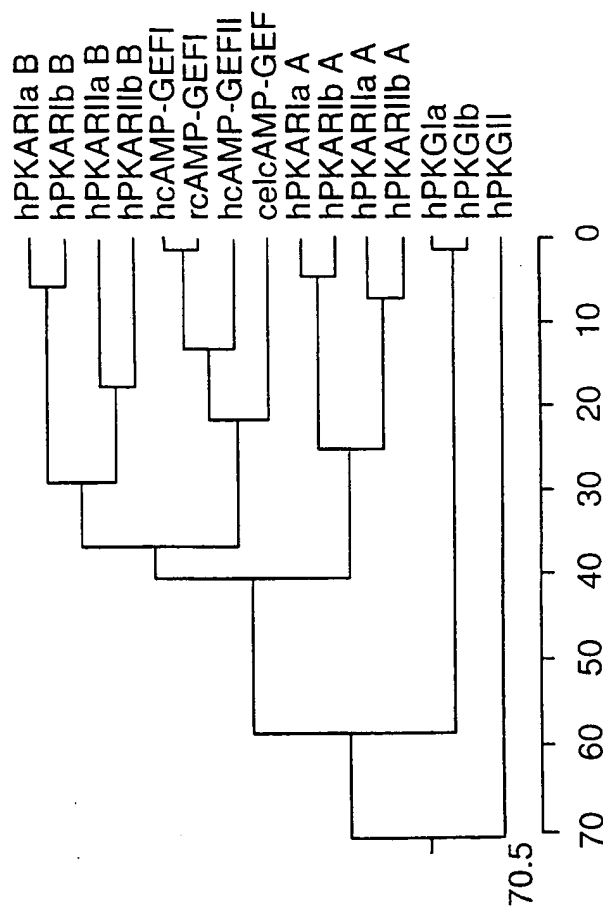


FIG. 3B



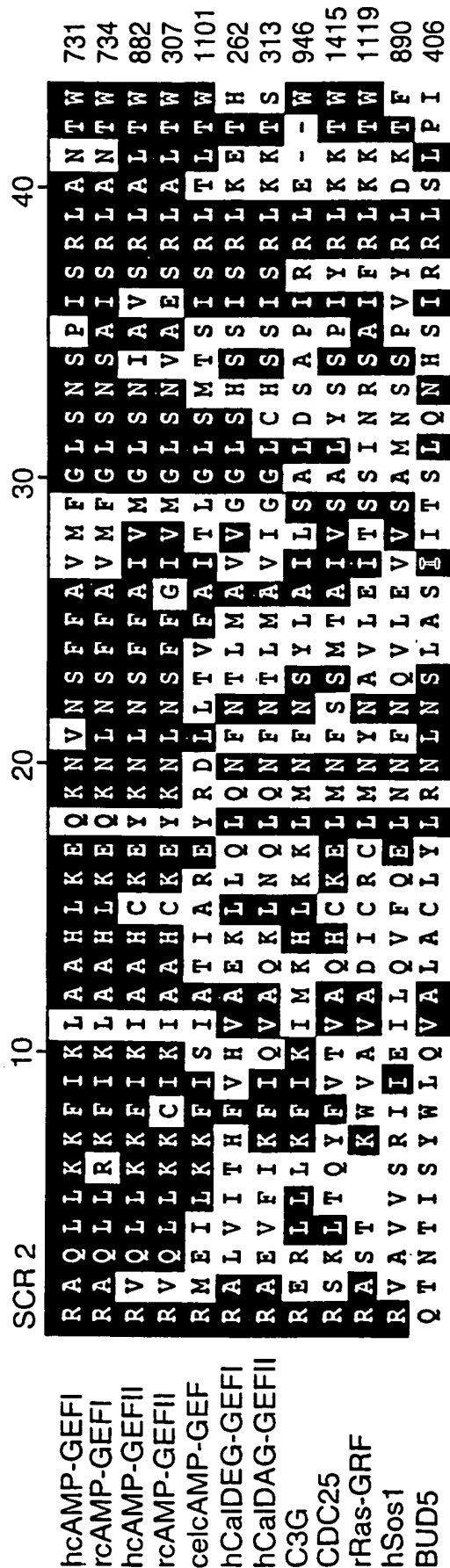
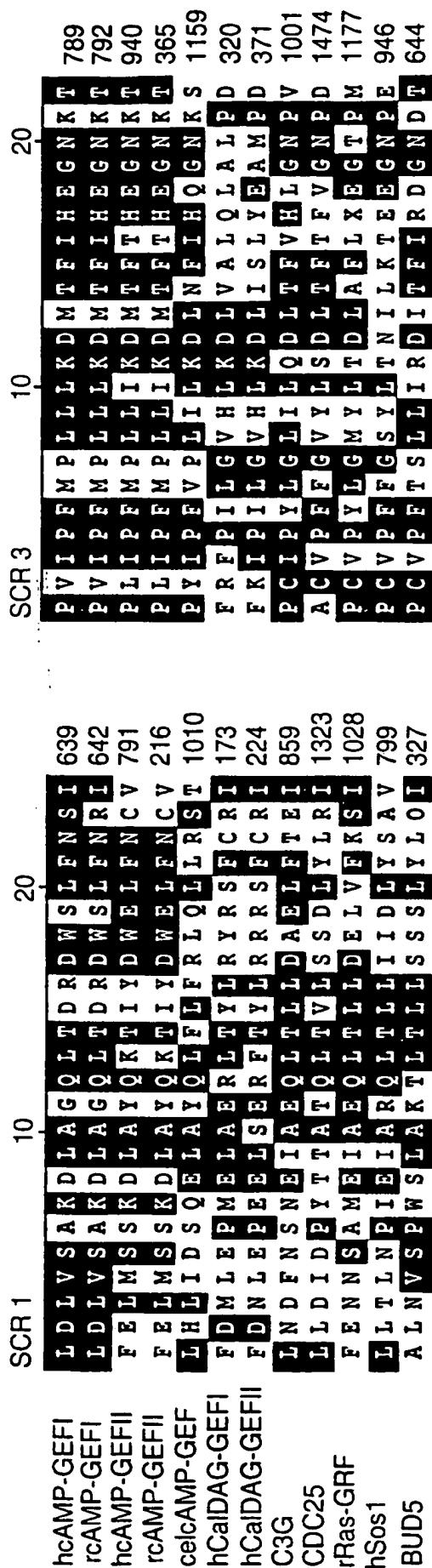


FIG. 3D

9/12

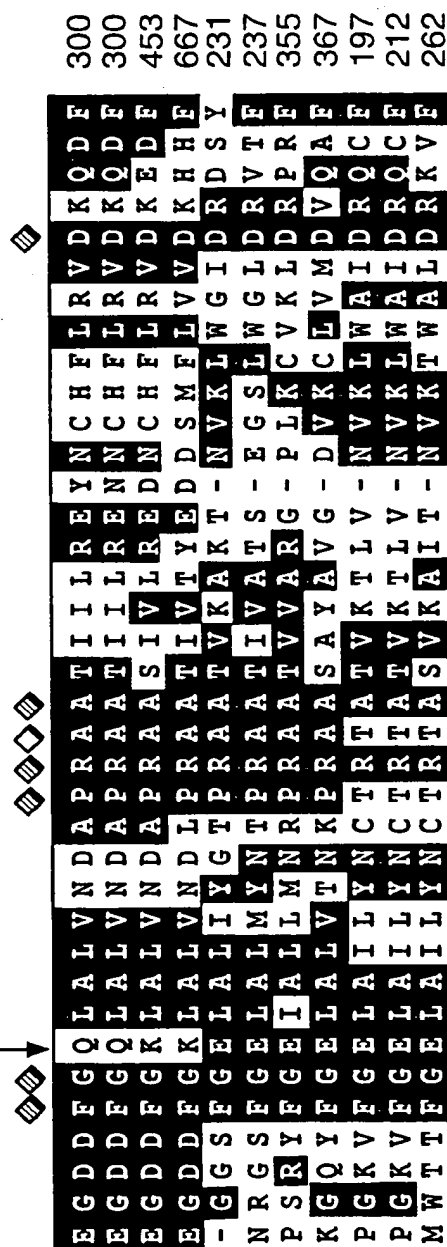
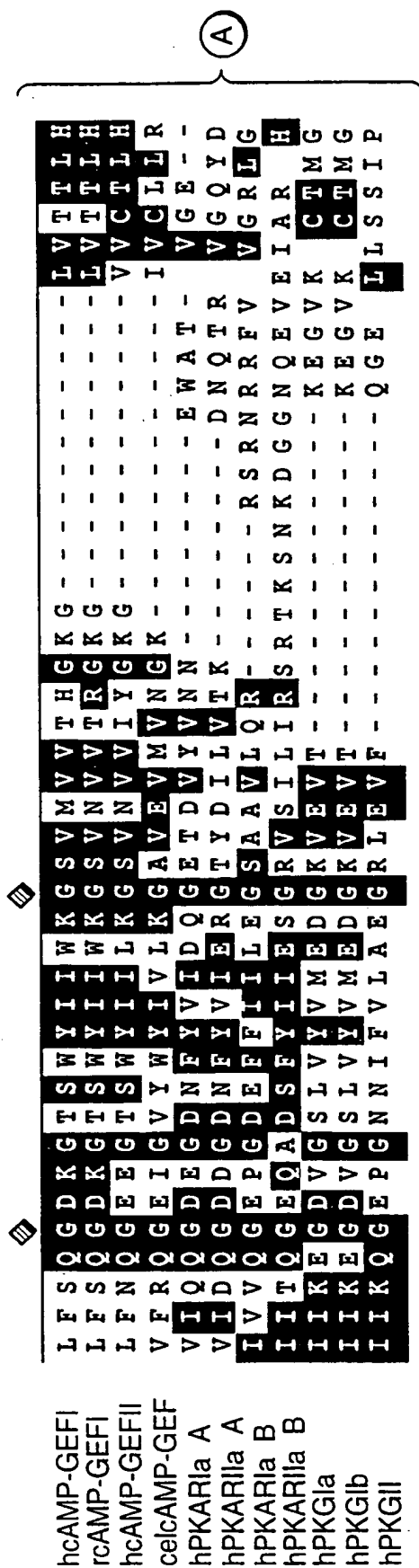


FIG. 3F-1	FIG. 3F-2	FIG. 3F-3
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FIG. 3F

FIG. 3E

10/12

hcAMP-GEFI	M V L - - - - -
hcAMP-GEFII	M V A A H A A H S S S S A E W I A C L D K
hcAMP-GEFI	- - - - - H Q H P
hcAMP-GEFII	Y A V L A G S L D V K V S E T S S H Q D A
hcAMP-GEFI	- - - - - T P
hcAMP-GEFII	P Y G V M E T G S M M D R I P D K K N T P
hcAMP-GEFI	L V D G I L A L G L G V H S R S Q V V G I
hcAMP-GEFII	L V D W M M Q Q T P C V H S R T Q A V G M
hcAMP-GEFI	S Q R G P D A L L T V A L R K P P G Q R T
hcAMP-GEFII	S Q M G P D A M M R W I L R K P P G Q R T
hcAMP-GEFI	G S N K V V T N G K G L V T T L H K G D D
hcAMP-GEFII	G S N S V V I Y G K G V V C T L H K G D D
hcAMP-GEFI	A S Q G A - G P S R P P T P G R N R Y T V
hcAMP-GEFII	V P A G N R R S N Q G N S Q P Q Q K Y T V
hcAMP-GEFI	G G S E Q E R S T Y V C M E R Q Q I L R L
hcAMP-GEFII	Q G T E Q E K K D Y A L M N K K K V I R L
hcAMP-GEFI	S P Q K K A R M L P V W L P N Q D K P L P
hcAMP-GEFII	A P Q R K H K V L L Q Q F N T G D K R - A
hcAMP-GEFI	G D A I G L Q P D A R G V A T S L G L N E
hcAMP-GEFII	G K K V V L K P N D V S V F T F L T I N G
hcAMP-GEFI	H Y V L G P Q H L R D V T T A K L E R F N
hcAMP-GEFII	Y M T F Q R M N F K K - T T A K L D L F L
hcAMP-GEFI	R L A M T W E R L P H K V R K L Y S A L K
hcAMP-GEFII	R L A L T W E K L P S K F K K F Y A E F K
hcAMP-GEFI	A A R M L H H C R S R N P V P L S F L R S
hcAMP-GEFII	T A R T V R Y Y R S Q - - - P F N F - - -

FIG. 3F-1



12/12

- - - C S Y Q L L L K - - - - -	20
I C L C G Y Y E N L K K G I T L F R Q G D I G T N W	80
- L R - - - - - W - - - - -	32
L L R I E Q K D F K A L W E K Y R Q Y M A G L L A P	160
L A T C P N L I R D R K Y H L R L Y R Q C C S G R E	92
L S R A P H M I R D R K Y H L K T Y R Q C C V G T E	240
F E - - P V G T H E M - - - - E E E L A E A V A L L	166
H E D A P L P T E E E K K E C D E E L Q D T M L L L	319
F E P H S K A G T V L F S Q G D K G T S W Y X X W K	246
F E S H A K G G T V L F N Q G E E G T S W Y X X L K	399
N R I I K D V E A K T M K L E G K G K V V L V L K R	326
N R I L R D V E A N T V K L K G K D Q D V L V L K K	479
L L T H R V F M P S A Q L C A A L L H H F H V E P A	405
I M M H C V F M P N T Q L C P A L V A H Y H A Q P S	559
R L S N L L R E Q W P E R R R C H R L E N G C G M A	485
R M I A A L K E Q L P E L E K I V K Q I S E - D A K	638
V R E V M A A L A Q E D G W T K G Q V L V K V N S A	565
V K E V I S A V A D K L G S G E G L I I V K M S S G	717
S A K D L A G Q L T D H D W S L F N S I H Q V E L I	645
S S K D L A Y Q M T I Y D W E L F N C V H R L E L I	797
A A H L R R Q K N V N S F F A V M F G L G K S P I S	725
A A H C R R Y K N L N S F F A I V X G L G K I A V S	876
M T F I H E G N H T L W E N L I N F G R M R N M A R	805
M T F T H E G N K T F I D N L V N F G R M R N I A N	956
V Q Q L K V I D N Q R E L S R L S R E L E P	881
V R Q L N V I D N Q R T L S Q N S H R L E P R R F	1011

FIG. 3F-3

SUBSTITUTE SHEET (RULE 26)

## SEQUENCE LISTING

<110> Kawasaki, Hiroaki  
Graybiel, Ann  
Housman, David

<120> Genes Integrating Signal Transduction Pathways

<130> MIT-103

<140>

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<150> US 60/105,507

<151> 1998-10-23

<150> US 60/108,685

<151> 1998-11-16

<160> 18

<170> PatentIn Ver. 2.0

<210> 1

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<222> (166)..(1989)

<223> CalDAG-GEFI

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cggggtttgca ttctgaagta aaggacttgg gacaggggta cgaatcgagc actgtgggag 120

gctctgagag tgtaacttgg gtctagccca ctggcaccgg cagcc atg gcg agc act 177  
Met Ala Ser Thr  
1

ctg gac ctg gac aag ggt tgc acc gtg gag gag ctg ctc cgt ggc tgt 225  
Leu Asp Leu Asp Lys Gly Cys Thr Val Glu Glu Leu Leu Arg Gly Cys  
5 10 15 20

atc gaa gcc ttt gat gac tct gga aag gtg cga gat cca cag cta gtg 273  
Ile Glu Ala Phe Asp Ser Gly Lys Val Arg Asp Pro Gln Leu Val  
25 30 35

cgc atg ttt ctc atg atg cac ccc tgg tac ata cct tcc tct cag ctg 321  
Arg Met Phe Leu Met Met His Pro Trp Tyr Ile Pro Ser Ser Gln Leu  
40 45 50

gct tcg aaa ctg ctc cac ttc tat cag caa tcc cgg aag gac aac tcc 369  
Ala Ser Lys Leu Leu His Phe Tyr Gln Gln Ser Arg Lys Asp Asn Ser  
55 60 65

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Asn Ser Leu Gln Val Lys Thr Cys His Leu Val Arg Tyr Trp Val Ser  
70 75 80

gcc ttc cca gca gag ttc gac ttg aac cca gag ctg gct gaa ccg atc 465  
Ala Phe Pro Ala Glu Phe Asp Leu Asn Pro Glu Leu Ala Glu Pro Ile  
85 90 95 100

aag gag ctg aag gct ctg tta gac caa gaa gga aac cgc agg cac agc 513  
Lys Glu Leu Lys Ala Leu Leu Asp Gln Glu Gly Asn Arg Arg His Ser  
105 110 115

agc ctc atc gac atc gag agt gtc ccc acc tac aag tgg aag cgg cag 561  
Ser Leu Ile Asp Ile Glu Ser Val Pro Thr Tyr Lys Trp Lys Arg Gln  
120 125 130

gtg acc cag cgg aac cct gtg gaa cag aaa aag cgc aag atg tcc ctg 609  
Val Thr Gln Arg Asn Pro Val Glu Gln Lys Lys Arg Lys Met Ser Leu  
135 140 145

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Leu	Phe	Asp	His	Leu	Glu	Pro	Met	Glu	Leu	Ala	Glu	His	Leu	Thr	Tyr	
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Leu	Glu	Tyr	Arg	Ser	Phe	Cys	Lys	Ile	Leu	Phe	Gln	Asp	Tyr	His	Ser	
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Phe	Val	Thr	His	Gly	Cys	Thr	Val	Asp	Asn	Pro	Val	Leu	Glu	Arg	Phe	
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Ile	Ser	Leu	Phe	Asn	Ser	Val	Ser	Gln	Trp	Val	Gln	Leu	Met	Ile	Leu	
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Ser	Lys	Pro	Thr	Ala	Thr	Gln	Arg	Ala	Leu	Val	Ile	Thr	His	Phe	Val	
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cat	gtg	gca	gag	aag	ctg	ctg	cag	ctg	cag	aac	ttc	aac	acg	ttg	atg	897
His	Val	Ala	Glu	Lys	Leu	Gln	Gln	Leu	Gln	Asn	Phe	Asn	Thr	Leu	Met	
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gcc	gtc	gtg	gga	ggc	ctg	agc	cac	agc	tcc	atc	tca	cgc	ctc	aag	gag	945
Ala	Val	Val	Gly	Gly	Leu	Ser	His	Ser	Ser	Ile	Ser	Arg	Leu	Lys	Glu	
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Thr	His	Ser	His	Val	Ser	Pro	Asp	Thr	Ile	Lys	Leu	Trp	Glu	Gly	Leu	
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aca	gaa	cta	gtg	aca	gct	act	ggc	aac	tac	agc	aac	tac	cgg	cga	agg	1041
Thr	Glu	Leu	Val	Thr	Ala	Thr	Gly	Asn	Tyr	Ser	Asn	Tyr	Arg	Arg	Arg	
			280					285					290			
ctg	gcg	gcc	tgc	gtg	ggc	ttc	cgc	ttt	cct	atc	ctg	ggt	gtg	cac	ctc	1089
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Gly	Arg	Thr	Arg	Leu	Asn	Gly	Ala	Lys	Met	Arg	Gln	Leu	Phe	Ser	Ile	
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Leu	Glu	Glu	Leu	Ala	Met	Val	Thr	Ser	Leu	Arg	Pro	Pro	Val	Gln	Ala	
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Asn	Pro	Asp	Leu	Leu	Ser	Leu	Leu	Thr	Val	Ser	Leu	Asp	Gln	Tyr	Gln	
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Lys	Ser	Ser	Pro	Thr	Ser	Pro	Thr	Ser	Cys	Thr	Pro	Pro	Pro	Arg	Pro	
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cct	gtg	ctg	gaa	gag	tgg	acc	tca	gtt	gcc	aag	cct	aag	ctg	gac	caa	1425
Pro	Val	Leu	Glu	Glu	Trp	Thr	Ser	Val	Ala	Lys	Pro	Lys	Leu	Asp	Gln	
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gcc	ttg	gtg	gcc	gag	cac	att	gag	aag	atg	gtg	gag	tct	gtg	ttc	cgg	1473
Ala	Leu	Val	Ala	Glu	His	Ile	Glu	Lys	Met	Val	Glu	Ser	Val	Phe	Arg	
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Asn	Phe	Asp	Val	Asp	Gly	Asp	Gly	His	Ile	Ser	Gln	Glu	Glu	Phe	Gln	

- 3 -

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Ile	Ile	Arg	Gly	Asn	Phe	Pro	Tyr	Leu	Ser	Ala	Phe	Gly	Asp	Leu	Asp					
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cag	aac	cag	gat	ggc	tgc	atc	agc	cgg	gag	gag	atg	att	tcc	tac	ttc	1617				
Gln	Asn	Gln	Asp	Gly	Cys	Ile	Ser	Arg	Glu	Glu	Met	Ile	Ser	Tyr	Phe					
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Phe	Gln	Glu	Ser	Asn	Ser	Leu	Arg	Pro	Val	Ala	Cys	Arg	His	Cys	Lys					
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Ala	Leu	Ile	Leu	Gly	Ile	Tyr	Lys	Gln	Gly	Leu	Lys	Cys	Arg	Ala	Cys					
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ggt	gtg	aac	tgc	cac	aag	cag	tgc	aaa	gac	cga	ctg	tca	gtg	gaa	tgt	1809				
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		535				540						545								
cgc	cgc	cgc	gcc	cag	agt	gtg	agc	ctg	gag	ggc	tct	gca	ccc	tct	ccc	1857				
Arg	Arg	Arg	Ala	Gln	Ser	Val	Ser	Leu	Glu	Gly	Ser	Ala	Pro	Ser	Pro					
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Ser	Pro	Thr	His	Thr	His	His	Arg	Ala	Phe	Ser	Phe	Ser	Leu	Pro	Arg					
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Pro	Gly	Arg	Arg	Ser	Ser	Arg	Pro	Pro	Glu	Ile	Arg	Glu	Glu	Glu	Val					
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Gln	Thr	Val	Glu	Asp	Gly	Val	Phe	Asp	Ile	His	Leu									
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 35 40 45  
 Ser Ser Gln Leu Ala Ser Lys Leu Leu His Phe Tyr Gln Gln Ser Arg  
 50 55 60  
 Lys Asp Asn Ser Asn Ser Leu Gln Val Lys Thr Cys His Leu Val Arg  
 65 70 75 80  
 Tyr Trp Val Ser Ala Phe Pro Ala Glu Phe Asp Leu Asn Pro Glu Leu



95

**SUBSTITUTE SHEET (RULE 26)**

- 5 -

485 490 495

Phe Val His Asn Phe Gln Glu Ser Asn Ser Leu Arg Pro Val Ala Cys  
500 505 510

Arg His Cys Lys Ala Leu Ile Leu Gly Ile Tyr Lys Gln Gly Leu Lys  
515 520 525

Cys Arg Ala Cys Gly Val Asn Cys His Lys Gln Cys Lys Asp Arg Leu  
530 535 540

Ser Val Glu Cys Arg Arg Arg Ala Gln Ser Val Ser Leu Glu Gly Ser  
545 550 555 560

Ala Pro Ser Pro Ser Pro Thr His Thr His His Arg Ala Phe Ser Phe  
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Met Ala Gly Thr Leu  
1 5

gac ctg gac aag ggc tgc acg gtg gag gag ctg ctc cgc ggc tgc atc 223  
Asp Leu Asp Lys Gly Cys Thr Val Glu Glu Leu Leu Arg Gly Cys Ile  
10 15 20

gaa gcc ttc gat gac tcc ggg aag gtg cgg gac ccg cag ctg gtg cgc 271  
Glu Ala Phe Asp Asp Ser Gly Lys Val Arg Asp Pro Gln Leu Val Arg  
25 30 35

atg ttc ctc atg atg cac ccc tgg tac atc ccc tcc tct cag ctg gcg 319  
Met Phe Leu Met Met His Pro Trp Tyr Ile Pro Ser Ser Gln Leu Ala  
40 45 50

gcc aag ctg ctc cac atc tac caa caa tcc cgg aag gac aac tcc aat 367  
Ala Lys Leu Leu His Ile Tyr Gln Gln Ser Arg Lys Asp Asn Ser Asn  
55 60 65

tcc ctg cag gtg aaa acg tgc cac ctg gtc agg tac tgg atc tcc gcc 415  
Ser Leu Gln Val Lys Thr Cys His Leu Val Arg Tyr Trp Ile Ser Ala  
70 75 80 85

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90 95 100

gag ctg aag gct ctg cta gac caa gaa ggg aac cga cgg cac agc agc 511  
Glu Leu Lys Ala Leu Leu Asp Gln Glu Gly Asn Arg Arg His Ser Ser  
105 110 115

cta atc gac ata gac agc gtc cct acc tac aag tgg aag cgg cag gtg 559  
Leu Ile Asp Ile Asp Ser Val Pro Thr Tyr Lys Trp Lys Arg Gln Val  
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- 6 -

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ttt Phe 150	gac Asp	cac His	ctg Leu	gag Glu	ccc Pro 155	atg Met	gag Glu	ctg Leu	gcg Ala 160	gag Glu	cat His	ctc Leu	acc Thr	tac Tyr	ttg Leu 165	655
gag Glu	tat Tyr	cgc Arg	tcc Ser	ttc Phe 170	tgc Cys	aag Lys	atc Ile	ctg Leu	ttt Phe 175	cag Gln	gac Asp	tat Tyr	cac His	agt Ser 180	ttc Phe	703
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gtg Val 230	gag Ala	gag Glu	aag Lys	ctg Leu	cta Leu 235	cag Gln	ctg Leu	cag Gln	aac Asn	ttc Phe 240	aac Asn	acg Thr	ctg Leu	atg Met	gca Ala 245	895
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gac Asp 310	ctg Leu	gtg Val	gcc Ala	ctg Leu	cag Gln 315	ctg Leu	gca Ala	ctg Leu	cct Pro	gac Asp 320	tgg Trp	ctg Leu	gac Asp	cca Pro	gcc Ala 325	1135
cgg Arg	acc Thr	cgg Arg	ctc Leu	aac Asn 330	ggg Gly	gcc Ala	aag Lys	atg Met	aag Lys 335	cag Gln	ctc Leu	ttt Phe	agc Ser	atc Ile 340	ctg Leu	1183
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ctc Leu	gtg Val	gtg Val	gag Glu 425	cac His	atc Ile	gag Glu	aag Lys	atg Met 430	gtg Val	gag Glu	tct Ser	gtg Val	ttc Phe 435	cgg Arg	aac Asn	1471

- 7 -

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 aac cag gat ggc tgc atc agc agg gag gag atg gtt tcc tat ttc ctg 1615  
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 Arg Ser Ser Ser Val Leu Gly Gly Arg Met Gly Phe Val His Asn Phe  
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 Pro Met His Ser His His His Arg Ala Phe Ser Phe Ser Leu Pro Arg  
 570 575 580  
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 Pro Gly Arg Arg Gly Ser Arg Pro Pro Glu Ile Arg Glu Glu Glu Val  
 585 590 595  
 cag acg gtg gag gat ggg gtg ttt gac atc cac ttg taatagatgc 1997  
 Gln Thr Val Glu Asp Gly Val Phe Asp Ile His Leu  
 600 605

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 Ser Ser Gln Leu Ala Ala Lys Leu Leu His Ile Tyr Gln Gln Ser Arg  
 50 55 60  
 Lys Asp Asn Ser Asn Ser Leu Gln Val Lys Thr Cys His Leu Val Arg  
 65 70 75 80

Tyr	Trp	Ile	Ser	Ala 85	Phe	Pro	Ala	Glu	Phe 90	Asp	Leu	Asn	Pro	Glu 95	Leu
Ala	Glu	Gln	Ile 100	Lys	Glu	Leu	Lys	Ala 105	Leu	Leu	Asp	Gln	Glu 110	Gly	Asn
Arg	Arg	His 115	Ser	Ser	Leu	Ile	Asp 120	Ile	Asp	Ser	Val	Pro 125	Thr	Tyr	Lys
Trp	Lys 130	Arg	Gln	Val	Thr	Gln 135	Arg	Asn	Pro	Val	Gly 140	Gln	Lys	Lys	Arg
Lys 145	Met	Ser	Leu	Leu	Phe 150	Asp	His	Leu	Glu	Pro 155	Met	Glu	Leu	Ala	Glu 160
His	Leu	Thr	Tyr	Leu 165	Glu	Tyr	Arg	Ser	Phe 170	Cys	Lys	Ile	Leu	Phe 175	Gln
Asp	Tyr	His	Ser 180	Phe	Val	Thr	His	Gly 185	Cys	Thr	Val	Asp	Asn 190	Pro	Val
Leu	Glu	Arg 195	Phe	Ile	Ser	Leu	Phe 200	Asn	Ser	Val	Ser	Gln 205	Trp	Val	Gln
Leu	Met 210	Ile	Leu	Ser	Lys	Pro 215	Thr	Ala	Pro	Gln	Arg 220	Ala	Leu	Val	Ile
Thr 225	His	Phe	Val	His 230	Val	Ala	Glu	Lys	Leu	Leu 235	Gln	Leu	Gln	Asn	Phe 240
Asn	Thr	Leu	Met	Ala 245	Val	Val	Gly	Gly	Leu 250	Ser	His	Ser	Ser	Ile 255	Ser
Arg	Leu	Lys	Glu 260	Thr	His	Ser	His	Val 265	Ser	Pro	Glu	Thr	Ile 270	Lys	Leu
Trp	Glu	Gly 275	Leu	Thr	Glu	Leu	Val 280	Thr	Ala	Thr	Gly	Asn 285	Tyr	Gly	Asn
Tyr	Arg 290	Arg	Arg	Leu	Ala	Ala 295	Cys	Val	Gly	Phe	Arg 300	Phe	Pro	Ile	Leu
Gly 305	Val	His	Leu	Lys	Asp 310	Leu	Val	Ala	Leu	Gln 315	Leu	Ala	Leu	Pro	Asp 320
Trp	Leu	Asp	Pro	Ala 325	Arg	Thr	Arg	Leu	Asn 330	Gly	Ala	Lys	Met	Lys 335	Gln
Leu	Phe	Ser	Ile 340	Leu	Glu	Glu	Leu	Ala 345	Met	Val	Thr	Ser	Leu 350	Arg	Pro
Pro	Val	Gln 355	Ala	Asn	Pro	Asp	Leu 360	Leu	Ser	Leu	Leu	Thr 365	Val	Ser	Leu
Asp	Gln 370	Tyr	Gln	Thr	Glu	Asp 375	Glu	Leu	Tyr	Gln	Leu 380	Ser	Leu	Gln	Arg
Glu 385	Pro	Arg	Ser	Lys	Ser 390	Ser	Pro	Thr	Ser	Pro 395	Thr	Ser	Cys	Thr	Pro 400
Pro	Pro	Arg	Pro	Pro 405	Val	Leu	Glu	Glu	Trp 410	Thr	Ser	Ala	Ala	Lys 415	Pro
Lys	Leu	Asp	Gln 420	Ala	Leu	Val	Val	Glu 425	His	Ile	Glu	Lys	Met 430	Val	Glu
Ser	Val	Phe 435	Arg	Asn	Phe	Asp	Val 440	Asp	Gly	Asp	Gly	His 445	Ile	Ser	Gln
Glu	Glu 450	Phe	Gln	Ile	Ile	Arg 455	Gly	Asn	Phe	Pro	Tyr 460	Leu	Ser	Ala	Phe
Gly 465	Asp	Leu	Asp	Gln 470	Asn	Gln	Asp	Gly	Cys	Ile 475	Ser	Arg	Glu	Glu	Met 480

- 9 -

Val Ser Tyr Phe Leu Arg Ser Ser Ser Val Leu Gly Gly Arg Met Gly  
 485 490 495  
 Phe Val His Asn Phe Gln Glu Ser Asn Ser Leu Arg Pro Val Ala Cys  
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 Arg His Cys Lys Ala Leu Ile Leu Gly Ile Tyr Lys Gln Gly Leu Lys  
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 Cys Arg Ala Cys Gly Val Asn Cys His Lys Gln Cys Lys Asp Arg Leu  
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 Ser Val Glu Cys Arg Arg Arg Ala Gln Ser Val Ser Leu Glu Gly Ser  
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 Ala Pro Ser Pro Ser Pro Met His Ser His His His Arg Ala Phe Ser  
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 Met Gly Thr Leu Gly Lys Ala Arg Glu  
 1 5  
 gct ccg cgg aaa cct tgc cat ggc tcc aga gct ggc ccc aaa gga aga 219  
 Ala Pro Arg Lys Pro Cys His Gly Ser Arg Ala Gly Pro Lys Gly Arg 25  
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 cta gag gcc aaa tca acc aac agt cct ctc cct gcc cag ccc agc ttg 267  
 Leu Glu Ala Lys Ser Thr Asn Ser Pro Leu Pro Ala Gln Pro Ser Leu 40  
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 gcc cag atc acc cag ttc cga atg atg gtg tcc ctg gga cat ctg gcc 315  
 Ala Gln Ile Thr Gln Phe Arg Met Met Val Ser Leu Gly His Leu Ala 55  
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 aaa gga gcc agc ctg gat gat ctt att gac agc tgc att caa tct ttc 363  
 Lys Gly Ala Ser Leu Asp Asp Leu Ile Asp Ser Cys Ile Gln Ser Phe 60 65 70  
 60 65 70  
 gat gca gat gga aac ctg tgt cga agt aac cag ctg tta caa gtc atg 411  
 Asp Ala Asp Gly Asn Leu Cys Arg Ser Asn Gln Leu Leu Gln Val Met 75 80 85  
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 cta acc atg cac cga atc atc atc tcc tcg gcc gag ctg ctg caa aaa 459  
 Leu Thr Met His Arg Ile Ile Ile Ser Ser Ala Glu Leu Leu Gln Lys 90 95 100 105  
 90 95 100 105  
 ctc atg aat cta tat aag gac gcc ctg gaa aag aat tct cca gga att 507  
 Leu Met Asn Leu Tyr Lys Asp Ala Leu Glu Lys Asn Ser Pro Gly Ile 110 115 120  
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- 10 -

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Cys	Leu	Lys	Ile	Cys	Tyr	Phe	Val	Arg	Tyr	Trp	Ile	Thr	Glu	Phe	Trp	
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Ile	Met	Phe	Lys	Met	Asp	Ala	Ser	Leu	Thr	Ser	Thr	Met	Glu	Glu	Phe	
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cag	gac	ctg	gtg	aaa	gcc	aat	ggt	gag	gag	tcc	cac	tgc	cac	ctc	atc	651
Gln	Asp	Leu	Val	Lys	Ala	Asn	Gly	Glu	Glu	Ser	His	Cys	His	Leu	Ile	
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gac	acg	aca	caa	att	aat	tct	cga	gac	tgg	tcc	agg	aaa	ctg	act	cag	699
Asp	Thr	Thr	Gln	Ile	Asn	Ser	Arg	Asp	Trp	Ser	Arg	Lys	Leu	Thr	Gln	
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agg	ata	aaa	tca	aat	acc	agc	aag	aag	cgg	aaa	gtg	tcc	ctg	ctg	ttt	747
Arg	Ile	Lys	Ser	Asn	Thr	Ser	Lys	Lys	Arg	Lys	Val	Ser	Leu	Leu	Phe	
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gac	cat	ctt	gaa	cct	gaa	gaa	ctg	tct	gaa	cac	ctc	acc	tac	ctt	gag	795
Asp	His	Leu	Glu	Pro	Glu	Glu	Leu	Ser	Glu	His	Leu	Thr	Tyr	Leu	Glu	
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Phe	Lys	Ser	Phe	Arg	Arg	Ile	Ser	Phe	Ser	Asp	Tyr	Gln	Asn	Tyr	Leu	
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Val	Asn	Ser	Cys	Val	Lys	Glu	Asn	Pro	Thr	Met	Glu	Arg	Ser	Ile	Ala	
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Leu	Cys	Asn	Gly	Ile	Ser	Gln	Trp	Val	Gln	Leu	Met	Val	Leu	Ser	Arg	
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ccc	acc	cca	cag	ctc	cgg	gca	gag	gtc	ttc	atc	aag	ttc	atc	cat	gtg	987
Pro	Thr	Pro	Gln	Leu	Arg	Ala	Glu	Val	Phe	Ile	Lys	Phe	Ile	His	Val	
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gct	cag	aag	ctc	cac	cag	cta	cag	aac	ttc	aac	acg	cta	atg	gct	gtg	1035
Ala	Gln	Lys	Leu	His	Gln	Leu	Gln	Asn	Phe	Asn	Thr	Leu	Met	Ala	Val	
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atc	ggg	gga	ctg	tgt	cac	agc	tcc	atc	tcc	agg	ctc	aag	gag	aca	agt	1083
Ile	Gly	Gly	Leu	Cys	His	Ser	Ser	Ile	Ser	Arg	Leu	Lys	Glu	Thr	Ser	
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Ser	His	Val	Pro	His	Glu	Ile	Asn	Lys	Val	Leu	Gly	Glu	Met	Thr	Glu	
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ctg	ctg	tcc	tcc	tgc	aga	aac	tat	gac	aac	tac	agg	cga	gcc	tat	ggt	1179
Leu	Leu	Ser	Ser	Cys	Arg	Asn	Tyr	Asp	Asn	Tyr	Arg	Arg	Ala	Tyr	Gly	
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gag	tgc	acc	cac	ttc	aaa	atc	ccc	ata	ctg	ggt	gtg	cac	ctc	aag	gac	1227
Glu	Cys	Thr	His	Phe	Lys	Ile	Pro	Ile	Leu	Gly	Val	His	Leu	Lys	Asp	
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ctc	ata	tcc	cta	tat	gaa	gcc	atg	cct	gac	tac	ctg	gaa	gac	ggg	aag	1275
Leu	Ile	Ser	Leu	Tyr	Glu	Ala	Met	Pro	Asp	Tyr	Leu	Glu	Asp	Gly	Lys	
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gtg	aat	gtc	caa	aag	ctc	ctg	gcc	ctt	tac	aat	cac	atc	aat	gag	ttg	1323
Val	Asn	Val	Gln	Lys	Leu	Leu	Ala	Leu	Tyr	Asn	His	Ile	Asn	Glu	Leu	
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gtc	cag	ctg	cag	gac	gtg	gcc	cca	cca	ttg	gat	gcc	aac	aag	gac	ctg	1371
Val	Gln	Leu	Gln	Asp	Val	Ala	Pro	Pro	Leu	Asp	Ala	Asn	Lys	Asp	Leu	
		395				400					405					
gtg	cac	ctg	ctg	acg	tta	tcc	ctg	gat	cta	tac	tac	acc	gaa	gat	gaa	1419
Val	His	Leu	Leu	Thr	Leu	Ser	Leu	Asp	Leu	Tyr	Tyr	Thr	Glu	Asp	Glu	
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- 11 -

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Pro	Leu	Thr	Pro	Ser	Lys	Pro	Pro	Val	Val	Val	Asp	Trp	Ala	Ser	Gly	
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Val	Ser	Pro	Lys	Pro	Asp	Pro	Lys	Thr	Ile	Ser	Lys	His	Val	Gln	Arg	
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Met	Val	Asp	Ser	Val	Phe	Lys	Asn	Tyr	Asp	Leu	Asp	Gln	Asp	Gly	Tyr	
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Phe	Cys	Val	Met	Asp	Lys	Asp	Arg	Glu	Gly	Leu	Ile	Ser	Arg	Asp	Glu	
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Ile	Thr	Ala	Tyr	Phe	Met	Arg	Ala	Ser	Ser	Ile	Tyr	Ser	Lys	Leu	Gly	
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Leu	Gly	Phe	Pro	His	Asn	Phe	Gln	Glu	Thr	Thr	Tyr	Leu	Lys	Pro	Thr	
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Phe	Cys	Asp	Asn	Cys	Ala	Gly	Phe	Leu	Trp	Gly	Val	Ile	Lys	Gln	Gly	
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Tyr	Arg	Cys	Lys	Asp	Cys	Gly	Met	Asn	Cys	His	Lys	Gln	Cys	Lys	Asp	
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ctg	gta	gtg	ttt	gag	tgc	aag	aaa	cga	tcc	aag	agc	ccg	gcg	gta	tcc	1947
Leu	Val	Val	Phe	Glu	Cys	Lys	Lys	Arg	Ser	Lys	Ser	Pro	Ala	Val	Ser	
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Thr	Glu	Asn	Ile	Ser	Ser	Val	Val	Pro	Met	Ser	Thr	Leu	Cys	Pro	Leu	
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Gly	Thr	Lys	Asp	Leu	Leu	His	Ala	Pro	Glu	Glu	Gly	Ser	Phe	Ile	Phe	
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cag	aat	gga	gag	gtt	gtg	gac	cac	agt	gag	gag	agc	aag	gat	agg	acc	2091
Gln	Asn	Gly	Glu	Val	Val	Asp	His	Ser	Glu	Glu	Ser	Lys	Asp	Arg	Thr	
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Ile	Met	Leu	Leu	Gly	Val	Ser	Ser	Gln	Lys	Ile	Ser	Val	Arg	Leu	Lys	
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Arg	Thr	Val	Ala	His	Lys	Thr	Thr	Gln	Thr	Glu	Ser	Phe	Pro	Trp	Val	
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ggc	ggc	gag	atg	ccc	cct	ggt	cac	ttt	gtg	ctg	act	tct	cca	aga	aag	2235
Gly	Gly	Glu	Met	Pro	Pro	Gly	His	Phe	Val	Leu	Thr	Ser	Pro	Arg	Lys	
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tca	gca	caa	ggg	gct	ctt	tat	gtg	cac	agt	cca	gca	tct	ccg	tgc	ccc	2283
Ser	Ala	Gln	Gly	Ala	Leu	Tyr	Val	His	Ser	Pro	Ala	Ser	Pro	Cys	Pro	
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agc	cca	gca	ctg	gtc	cgg	aag	cgg	gca	ttc	gtc	aag	tgg	gag	aac	aaa	2331
Ser	Pro	Ala	Leu	Val	Arg	Lys	Arg	Ala	Phe	Val	Lys	Trp	Glu	Asn	Lys	



- 12 -

715                      720                      725

gag tcc ctt atc aaa cca aaa cca gag ctt cac ctc agg ctc cgg acc 2379  
 Glu Ser Leu Ile Lys Pro Lys Pro Glu Leu His Leu Arg Leu Arg Thr  
 730                      735                      740                      745

tac caa gaa ctg gaa cag gag gta aat acc ctg agg gca gat aac gat 2427  
 Tyr Gln Glu Leu Glu Gln Glu Val Asn Thr Leu Arg Ala Asp Asn Asp  
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gct ctg aag atc cag ctg aag tat gca cag aaa caa ata gaa tcc ctg 2475  
 Ala Leu Lys Ile Gln Leu Lys Tyr Ala Gln Lys Gln Ile Glu Ser Leu  
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cag ctt ggc aaa agc aat cac gtc tta gca cag atg gac cac ggt gat 2523  
 Gln Leu Gly Lys Ser Asn His Val Leu Ala Gln Met Asp His Gly Asp  
                     780                      785                      790

ggt act taatccagaa attcaaggaa cagaatctgc agacggggttt actgggatct 2579  
 Gly Thr  
                     795

cacttcaaaa ctgattgcag aggttcagca acttttagact gattgacttt taaagggcag 2639

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Ser Pro Leu Pro Ala Gln Pro Ser Leu Ala Gln Ile Thr Gln Phe Arg  
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Met Met Val Ser Leu Gly His Leu Ala Lys Gly Ala Ser Leu Asp Asp

- 13 -

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Arg Ser Asn Gln 85	Leu Gln Val Met 90	Leu Thr Met His Arg Ile Ile 95
Ile Ser Ser 100	Ala Glu Leu Leu Gln Lys 105	Leu Met Asn Leu Tyr Lys Asp 110
Ala Leu Glu 115	Lys Asn Ser Pro Gly Ile Cys 120	Leu Lys Ile Cys Tyr Phe 125
Val Arg Tyr Trp 130	Ile Thr Glu Phe Trp Ile Met 135	Phe Lys Met Asp Ala 140
Ser Leu Thr Ser Thr 145	Met Glu Glu Phe Gln Asp 150 155	Leu Val Lys Ala Asn 160
Gly Glu Glu Ser 165	His Cys His Leu Ile Asp 170	Thr Thr Gln Ile Asn Ser 175
Arg Asp Trp 180	Ser Arg Lys Leu Thr Gln Arg 185	Ile Lys Ser Asn Thr Ser 190
Lys Lys Arg 195	Lys Val Ser Leu Leu Phe Asp 200	His Leu Glu Pro Glu Glu 205
Leu Ser Glu His 210	Leu Thr Tyr Leu Glu Phe Lys 215	Ser Phe Arg Arg Ile 220
Ser Phe Ser Asp Tyr 225	Gln Asn Tyr Leu Val Asn Ser 230 235	Cys Val Lys Glu 240
Asn Pro Thr Met 245	Glu Arg Ser Ile Ala Leu Cys 250	Asn Gly Ile Ser Gln 255
Trp Val Gln 260	Leu Met Val Leu Ser Arg 265	Pro Thr Pro Gln Leu Arg Ala 270
Glu Val Phe 275	Ile Lys Phe Ile His Val Ala 280	Gln Lys Leu His Gln Leu 285
Gln Asn Phe Asn Thr 290	Leu Met Ala Val Ile Gly 295	Gly Gly Leu Cys His Ser 300
Ser Ile Ser Arg 305	Leu Lys Glu Thr Ser Ser His 310 315	Val Pro His Glu Ile 320
Asn Lys Val Leu 325	Gly Glu Met Thr Glu Leu Leu 330	Ser Ser Cys Arg Asn 335
Tyr Asp Asn Tyr 340	Arg Arg Ala Tyr Gly 345	Glu Cys Thr His Phe Lys Ile 350
Pro Ile Leu Gly 355	Val His Leu Lys Asp 360	Leu Ile Ser Leu Tyr Glu Ala 365
Met Pro Asp Tyr 370	Leu Glu Asp Gly Lys Val 375	Asn Val Gln Lys Leu Leu 380
Ala Leu Tyr Asn His 385	Ile Asn Glu Leu Val Gln 390 395	Leu Gln Asp Val Ala 400
Pro Pro Leu Asp 405	Ala Asn Lys Asp Leu Val 410	His Leu Leu Thr Leu Ser 415
Leu Asp Leu Tyr 420	Tyr Thr Glu Asp Glu Ile 425	Tyr Glu Leu Ser Tyr Ala 430
Arg Glu Pro Arg 435	Asn His Arg Ala Pro 440	Pro Leu Thr Pro Ser Lys Pro 445
Pro Val Val Val 450	Asp Trp Ala Ser Gly 455	Val Ser Pro Lys Pro Asp Pro 460

- 14 -

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Lys Ile Ala Ala Ser	Phe Pro Phe Ser Phe	Cys Val Met Asp Lys																		
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Arg Glu Gly Leu Ile	Ser Arg Asp Glu Ile Thr	Ala Tyr Phe Met Arg																		
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Ala Ser Ser Ile Tyr	Ser Lys Leu Gly Leu Gly	Phe Pro His Asn Phe																		
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	545			550											560					
Phe Leu Trp Gly Val	Ile Lys Gln Gly Tyr Arg	Cys Lys Asp Cys Gly																		
	565			570											575					
Met Asn Cys His Lys	Gln Cys Lys Asp Leu	Val Val Phe Glu Cys																		
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Lys Arg Ser Lys Ser	Pro Ala Val Ser Thr	Glu Asn Ile Ser Ser																		
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Val Pro Met Ser Thr	Leu Cys Pro Leu Gly	Thr Lys Asp Leu Leu																		
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Ala Pro Glu Glu Gly	Ser Phe Ile Phe Gln	Asn Gly Glu Val Val																		
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His Ser Glu Glu Ser	Lys Asp Arg Thr Ile	Met Leu Leu Gly Val																		
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Ser Gln Lys Ile Ser	Val Arg Leu Lys Arg	Thr Val Ala His Lys																		
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Thr Gln Thr Glu Ser	Phe Pro Trp Val Gly	Gly Glu Met Pro Pro																		
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His Phe Val Leu Thr	Ser Pro Arg Lys Ser	Ala Gln Gly Ala Leu																		
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Val His Ser Pro Ala	Ser Pro Cys Pro Ser	Pro Ala Leu Val Arg																		
	705			710											720					
Arg Ala Phe Val Lys	Trp Glu Asn Lys Glu	Ser Leu Ile Lys Pro																		
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Pro Glu Leu His Leu	Arg Leu Arg Thr Tyr	Gln Glu Leu Glu Gln																		
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Val Asn Thr Leu Arg	Ala Asp Asn Asp Ala	Leu Lys Ile Gln Leu																		
	755			760											765					
Tyr Ala Gln Lys Gln	Ile Glu Ser Leu Gln	Leu Gly Lys Ser Asn																		
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Met Gly Thr Leu																		
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Gly Lys Ala Arg Glu Ala Pro Arg Lys Pro Ser His Gly Cys Arg Ala																		20
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Ala Ser Lys Ala Arg Leu Glu Ala Lys Pro Ala Asn Ser Pro Phe Pro																		35
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Ser His Pro Ser Leu Ala His Ile Thr Gln Phe Arg Met Met Val Ser																		50
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ctg gga cat tta gcc aaa gga gcc agc ctg gac gat ctc att gac agc																		307
Leu Gly His Leu Ala Lys Gly Ala Ser Leu Asp Asp Leu Ile Asp Ser																		65
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Cys Ile Gln Ser Phe Asp Ala Asp Gly Asn Leu Cys Arg Ser Asn Gln																		75 80
70 85																		
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Leu Leu Gln Val Met Leu Thr Met His Arg Ile Val Ile Ser Ser Ala																		100
85 90																		
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Glu Leu Leu Gln Lys Val Ile Thr Leu Tyr Lys Asp Ala Leu Ala Lys																		105 110 115
110 115																		
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Asn Ser Pro Gly Leu Cys Leu Lys Ile Cys Tyr Phe Val Arg Tyr Trp																		120 125 130
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Ile Thr Glu Phe Trp Val Met Phe Lys Met Asp Ala Ser Leu Thr Asp																		135 140 145
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Thr Met Glu Glu Phe Gln Glu Leu Val Lys Ala Lys Gly Glu Glu Leu																		150 155 160
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His Cys Arg Leu Ile Asp Thr Thr Gln Ile Asn Ala Arg Asp Trp Ser																		165 170 175 180
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agg aaa ctt act caa agg ata aaa tca aat acc agc aag aaa cgg aaa																		691
Arg Lys Leu Thr Gln Arg Ile Lys Ser Asn Thr Ser Lys Lys Arg Lys																		185 190 195
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Val Ser Leu Leu Phe Asp His Leu Glu Pro Glu Glu Leu Ser Glu His																		200 205 210
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Leu Thr Tyr Leu Glu Phe Lys Ser Phe Arg Arg Ile Ser Phe Ser Asp																		215 220 225
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Tyr Gln Asn Tyr Leu Val Asn Ser Cys Val Lys Glu Asn Pro Thr Met																		230 235 240
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Glu Arg Ser Ile Ala Leu Cys Asn Gly Ile Ser Gln Trp Val Gln Leu																		245 250 255 260
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Met Val Leu Ser Arg Pro Thr Pro Gln Leu Arg Ala Glu Val Phe Ile																		265 270 275
265 270 275																		

- 16 -

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Thr Leu Met Ala Val Ile Gly Gly Leu Cys His Ser Ser Ile Ser Arg	
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Leu Lys Glu Thr Ser Ser His Val Pro His Glu Ile Asn Lys Val Leu	
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Gly Glu Met Thr Glu Leu Leu Ser Ser Ser Arg Asn Tyr Asp Asn Tyr	
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Arg Arg Ala Tyr Gly Glu Cys Thr Asp Phe Lys Ile Pro Ile Leu Gly	
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Val His Leu Lys Asp Leu Ile Ser Leu Tyr Glu Ala Met Pro Asp Tyr	
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Leu Gly Asp Gly Lys Val Asn Val His Lys Leu Leu Ala Leu Tyr Asn	
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Tyr Leu Lys Pro Thr Phe Cys Asp Asn Cys Ala Gly Phe Leu Trp Gly	
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- 17 -

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gga cct ttt aca ttc cct aat ggg gag gct gtg gaa cat ggt gag gag	Gly Pro Phe Thr Phe 630	Pro Asn Gly Glu Ala Val Glu His Gly Glu Glu	2035	
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- 18 -

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- 19 -

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Met Met Val Ser Leu Gly His Leu Ala Lys Gly Ala Ser Leu Asp Asp  
50 55 60  
Leu Ile Asp Ser Cys Ile Gln Ser Phe Asp Ala Asp Gly Asn Leu Cys  
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Arg Ser Asn Gln Leu Leu Gln Val Met Leu Thr Met His Arg Ile Val  
85 90 95  
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Val Arg Tyr Trp Ile Thr Glu Phe Trp Val Met Phe Lys Met Asp Ala  
130 135 140  
Ser Leu Thr Asp Thr Met Glu Glu Phe Gln Glu Leu Val Lys Ala Lys  
145 150 155 160  
Gly Glu Glu Leu His Cys Arg Leu Ile Asp Thr Thr Gln Ile Asn Ala  
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Arg Asp Trp Ser Arg Lys Leu Thr Gln Arg Ile Lys Ser Asn Thr Ser  
180 185 190  
Lys Lys Arg Lys Val Ser Leu Leu Phe Asp His Leu Glu Pro Glu Glu  
195 200 205  
Leu Ser Glu His Leu Thr Tyr Leu Glu Phe Lys Ser Phe Arg Arg Ile  
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245 250 255  
Trp Val Gln Leu Met Val Leu Ser Arg Pro Thr Pro Gln Leu Arg Ala  
260 265 270  
Glu Val Phe Ile Lys Phe Ile Gln Val Ala Gln Lys Leu His Gln Leu  
275 280 285  
Gln Asn Phe Asn Thr Leu Met Ala Val Ile Gly Gly Leu Cys His Ser  
290 295 300  
Ser Ile Ser Arg Leu Lys Glu Thr Ser Ser His Val Pro His Glu Ile  
305 310 315 320  
Asn Lys Val Leu Gly Glu Met Thr Glu Leu Leu Ser Ser Ser Arg Asn  
325 330 335  
Tyr Asp Asn Tyr Arg Arg Ala Tyr Gly Glu Cys Thr Asp Phe Lys Ile  
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Met Pro Asp Tyr Leu Gly Asp Gly Lys Val Asn Val His Lys Leu Leu  
370 375 380  
Ala Leu Tyr Asn His Ile Ser Glu Leu Val Gln Leu Gln Glu Val Ala  
385 390 395 400



- 20 -

Pro	Pro	Leu	Glu	Ala	Asn	Lys	Asp	Leu	Val	His	Leu	Leu	Thr	Leu	Ser
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Leu	Asp	Leu	Tyr	Tyr	Thr	Glu	Asp	Glu	Ile	Tyr	Glu	Leu	Ser	Tyr	Ala
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Arg	Glu	Pro	Arg	Asn	His	Arg	Ala	Pro	Pro	Leu	Thr	Pro	Ser	Lys	Pro
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Pro	Val	Val	Val	Asp	Trp	Ala	Ser	Gly	Val	Ser	Pro	Lys	Pro	Asp	Pro
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Lys	Thr	Ile	Ser	Lys	His	Val	Gln	Arg	Met	Val	Asp	Ser	Val	Phe	Lys
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Asn	Tyr	Asp	His	Asp	Gln	Asp	Gly	Tyr	Ile	Ser	Gln	Glu	Glu	Phe	Glu
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Lys	Ile	Ala	Ala	Ser	Phe	Pro	Phe	Ser	Phe	Cys	Val	Met	Asp	Lys	Asp
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Arg	Glu	Gly	Leu	Ile	Ser	Arg	Asp	Glu	Ile	Thr	Ala	Tyr	Phe	Met	Arg
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Ala	Ser	Ser	Ile	Tyr	Ser	Lys	Leu	Gly	Leu	Gly	Phe	Pro	His	Asn	Phe
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Gln	Glu	Thr	Thr	Tyr	Leu	Lys	Pro	Thr	Phe	Cys	Asp	Asn	Cys	Ala	Gly
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Phe	Leu	Trp	Gly	Val	Ile	Lys	Gln	Gly	Tyr	Arg	Cys	Lys	Asp	Cys	Gly
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Met	Asn	Cys	His	Lys	Gln	Cys	Lys	Asp	Leu	Val	Val	Phe	Glu	Cys	Lys
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His	Gly	Glu	Glu	Ser	Lys	Asp	Arg	Thr	Ile	Met	Leu	Met	Gly	Val	Ser
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Lys	Arg	Ala	Phe	Val	Lys	Trp	Glu	Asn	Lys	Asp	Ser	Leu	Ile	Lys	Ser
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Lys	Glu	Glu	Leu	Arg	His	Leu	Arg	Leu	Pro	Thr	Tyr	Gln	Glu	Leu	Glu
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**SUBSTITUTE SHEET (RULE 26)**

- 22 -

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- 23 -

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cta Leu	gtg Val	agt Ser	gcc Ala	aag Lys 625	gac Asp	ctg Leu	gca Ala	ggc Gly	cag Gln 630	ctc Leu	aca Thr	gag Glu	cat His	gac Asp 635	tgg Trp	2104
aac Asn	ctc Leu	ttc Phe	aac Asn 640	agg Arg	atc Ile	cac His	cag Gln	gtg Val 645	gag Glu	ctg Leu	atc Ile	cac His	tat Tyr 650	gta Val	ctg Leu	2152
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ctc Leu	cac His	cac His	tgc Cys	cga Arg	agc Ser	cac His	agc Ser	acc Thr	gcg Ala	cct Pro	cta Leu	tca Ser	cca Pro	ctc Leu	aga Arg	2680

- 24 -

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Ser Arg Val Ser His Ile	His Glu Asp Ser Gln	Ala Ser Arg Ile Ser	
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aca tgt tcc gag cag tcc	ctg agc acc cgg agt	cca gcc agc acc tgg	2776
Thr Cys Ser Glu Gln Ser	Leu Ser Thr Arg Ser	Pro Ala Ser Thr Trp	
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gct tat gtc cag cag	ctg aag gtc att gac aac	cag cgg gaa ctg tcc	2824
Ala Tyr Val Gln Gln	Leu Lys Val Ile Asp Asn	Gln Arg Glu Leu Ser	
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Arg Leu Ser Arg Glu	Leu Glu Pro		
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Asp Trp Thr Phe Gln	Asp Arg Asp Ala Gln Phe	Tyr Arg Phe Pro Gly	
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Pro Glu Pro Gln Pro	Ala Gly Thr His Asp Val	Glu Glu Glu Leu Val	
	145	150	155
Glu Ala Met Ala Leu	Leu Ser Gln Arg Gly Pro	Asp Ala Leu Leu Thr	
	165	170	175

- 25 -

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 Asn Ser Val Lys Arg Glu Leu Ala Ala Val Leu Leu Phe Glu Pro His  
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 Ser Lys Ala Gly Thr Val Leu Phe Ser Gln Gly Asp Lys Gly Thr Ser  
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 Trp Tyr Ile Ile Trp Lys Gly Ser Val Asn Val Val Thr Arg Gly Lys  
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 Gly Leu Val Thr Thr Leu His Glu Gly Asp Asp Phe Gly Gln Leu Ala  
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 Asn Cys His Phe Leu Arg Val Asp Lys Gln Asp Phe Asn Arg Ile Ile  
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 Lys Asp Val Glu Ala Lys Thr Met Arg Leu Glu Glu His Gly Lys Val  
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 Val Phe Met Pro Cys Thr Gln Leu Phe Ala Ala Leu Leu His His Phe  
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 His Val Glu Pro Ser Glu Pro Ala Gly Gly Ser Glu Gln Glu Arg Ser  
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 Thr Tyr Ile Cys Asn Lys Arg Gln Gln Ile Leu Arg Leu Val Ser Arg  
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 Trp Val Ala Leu Tyr Ser Pro Met Leu Arg Ser Asp Pro Val Ala Thr  
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 Ser Phe Leu Gln Lys Leu Ser Asp Leu Val Ser Arg Asp Thr Arg Leu  
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 Ser Asn Leu Leu Arg Glu Gln Tyr Pro Glu Arg Arg Arg His His Arg  
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Glu	Ser	Leu	Asp	Phe	Ser	Glu	Ser	Leu	Glu	Gln	Ala	Ser	Thr	Glu	Arg							
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gtg	ctc	agg	gct	ggg	agg	cag	ctg	cat	cag	cat	cta	ctg	gcc	acc	tgc						425	
Val	Leu	Arg	Ala	Gly	Arg	Gln	Leu	His	Gln	His	Leu	Leu	Ala	Thr	Cys							
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cca	aac	ctc	atc	cga	gac	cgg	aag	tac	cac	ctt	agg	ctc	tat	cgg	cag						473	
Pro	Asn	Leu	Ile	Arg	Asp	Arg	Lys	Tyr	His	Leu	Arg	Leu	Tyr	Arg	Gln							
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tgc	tgc	tct	ggc	cgg	gag	ctg	gtg	gat	ggg	atc	ttg	gcc	ctg	gga	ctt						521	
Cys	Cys	Ser	Gly	Arg	Glu	Leu	Val	Asp	Gly	Ile	Leu	Ala	Leu	Gly	Leu							
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Gly	Val	His	Ser	Arg	Ser	Gln	Val	Val	Gly	Ile	Cys	Gln	Val	Leu	Leu							
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Gly	Thr</																					



- 28 -

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Thr	Met	Arg	Leu	Glu	Glu	His	Gly	Lys	Val	Val	Leu	Val	Leu	Glu	Arg	
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Ala	Ser	Gln	Gly	Ala	Gly	Pro	Ser	Arg	Pro	Pro	Thr	Pro	Gly	Arg	Asn	
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Arg	Tyr	Thr	Val	Met	Ser	Gly	Thr	Pro	Asp	Lys	Ile	Leu	Glu	Leu	Leu	
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Leu	Glu	Ala	Met	Gly	Leu	Asp	Ser	Ser	Ala	His	Asp	Pro	Lys	Glu	Thr	
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Gln	Leu	Cys	Ala	Ala	Leu	Leu	His	His	Phe	His	Val	Glu	Pro	Ala	Gly	
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Gly	Ser	Glu	Gln	Glu	Arg	Ser	Thr	Tyr	Val	Cys	Asn	Lys	Arg	Gln	Gln	
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Ile	Leu	Arg	Leu	Val	Ser	Gln	Trp	Val	Ala	Leu	Tyr	Gly	Ser	Met	Leu	
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Pro	Gln	Met	Lys	Ala	Arg	Asn	Leu	Pro	Val	Trp	Leu	Pro	Asn	Gln	Asp	
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- 29 -

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cta gcc cac acc tgg gag cgg ctg cct cac aaa gtc cgg aag ctg tac	Leu Ala His Thr Trp Glu Arg Leu Pro His Lys Val Arg Lys Leu Tyr	730					735					740			2441
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cca ctc aga agc cga gtt tcc cac ctc cac gag gac agc cag gtg gcg	Pro Leu Arg Ser Arg Val Ser His Leu His Glu Asp Ser Gln Val Ala	825					830						835		2729
agg att tcc aca tgc tcg gag cag tcc ctg agc acc cgg agt cca gcc	Arg Ile Ser Thr Cys Ser Glu Gln Ser Leu Ser Thr Arg Ser Pro Ala	840				845							850		2777
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- 30 -

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Trp Tyr Ile Ile Trp Lys Gly Ser Val Asn Val Val Thr His Gly Lys  
245 250 255

- 31 -

Gly Leu Val Thr Thr Leu His Glu Gly Asp Asp Phe Gly Gln Leu Ala  
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 Asn Cys His Phe Leu Arg Val Asp Lys Gln Asp Phe Asn Arg Ile Ile  
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 His Asp Pro Lys Glu Thr Phe Leu Ser Asp Phe Leu Leu Thr His Arg  
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 Trp Leu Pro Asn Gln Asp Glu Pro Leu Pro Gly Ser Ser Cys Ala Ile  
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 595 600 605  
 Thr Val Gly Ser Ala Glu Gly Leu Asp Leu Val Ser Ala Lys Asp Leu  
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 Gln Val Glu Leu Ile His Tyr Val Leu Gly Pro Gln His Leu Arg Asp  
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- 32 -

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Gln Tyr Trp Val Ala Thr Glu Leu Cys Leu Cys Pro Val Pro Gly Pro  
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Lys Val Arg Lys Leu Tyr Ser Ala Leu Glu Arg Leu Leu Asp Pro Ser  
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Trp Asn His Arg Val Tyr Arg Leu Ala Leu Ala Lys Leu Ser Pro Pro  
755 760 765  
Val Ile Pro Phe Met Pro Leu Leu Leu Lys Asp Met Thr Phe Ile His  
770 775 780  
Glu Gly Asn His Thr Leu Val Glu Asn Leu Ile Asn Phe Glu Lys Met  
785 790 795 800  
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Ser Thr Arg Ser Pro Ala Ser Thr Trp Ala Tyr Val Gln Gln Leu Lys  
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ggcaggtggg cctggctgtg gaggatagcc cagctctggg agcaccgctg gtgggagccc 180  
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Met Val Leu Arg Arg Met  
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cac cgg ccc cga agc tgc tcc tac cag ctg ctg ctg gag cac cag cat 281  
His Arg Pro Arg Ser Cys Ser Tyr Gln Leu Leu Leu Glu His Gln His  
10 15 20  
ccg agc tgc atc cag ggg ctg cgc tgg aca cca ctc acc aac agc gag 329  
Pro Ser Cys Ile Gln Gly Leu Arg Trp Thr Pro Leu Thr Asn Ser Glu  
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- 33 -

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			170					175					180			
cca Pro	ggt Gly	cag Gln	cgc Arg	acg Thr	gat Asp	gaa Glu	gag Glu	ctg Leu	gac Asp	ctc Leu	atc Ile	ttt Phe	gag Glu	gag Glu	ctg Leu	809
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ctg Leu	cac His	atc Ile	aag Lys	gct Ala	gtg Val	gcc Ala	cac His	ctc Leu	tcc Ser	aac Asn	tgc Ser	gtg Val	aag Lys	cga Arg	gaa Glu	857
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gga Gly	tct Ser	gtc Val	aac Asn	gtg Val	gtg Val	acc Thr	cat His	ggc Gly	aag Lys	ggg Gly	ctg Leu	gtg Val	acc Thr	acc Thr	ctg Leu	1001
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 Pro Leu Thr Asn Ser Glu Glu Ser Leu Asp Phe Ser Glu Ser Leu Glu  
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 Gln Ala Ser Thr Glu Arg Val Leu Arg Ala Gly Arg Gln Leu His Gln  
 50 55 60



- 36 -

His 65 Leu Leu Ala Thr Cys 70 Pro Asn Leu Ile Arg 75 Asp Arg Lys Tyr His 80  
 Leu Arg Leu Tyr Arg 85 Gln Cys Cys Ser Gly 90 Arg Glu Leu Val Asp 95 Gly  
 Ile Leu Ala Leu Gly 100 Leu Gly Val His 105 Ser Arg Ser Gln 110 Val Val Gly  
 Ile Cys Gln Val Leu Leu Asp 115 Glu Gly Ala Leu Cys His 125 Val Lys His  
 Asp Trp 130 Ala Phe Gln Asp 135 Arg Ala Gln Phe Tyr 140 Arg Phe Pro Gly  
 Pro Glu 145 Pro Glu Pro Val 150 Gly Thr His Glu Met 155 Glu Glu Glu Leu Ala 160  
 Glu Ala Val Ala Leu 165 Leu Ser Gln Arg Gly 170 Pro Asp Ala Leu Leu Thr 175  
 Val Ala Leu Arg 180 Lys Pro Pro Gly Gln 185 Arg Thr Asp Glu Glu 190 Leu Asp  
 Leu Ile Phe 195 Glu Glu Leu Leu His 200 Ile Lys Ala Val Ala His 205 Leu Ser  
 Asn Ser 210 Val Lys Arg Glu Leu 215 Ala Ala Val Leu Leu Phe Glu Pro His  
 Ser 225 Lys Ala Gly Thr Val 230 Leu Phe Ser Gln Gly 235 Asp Lys Gly Thr Ser 240  
 Trp Tyr Ile Ile Trp 245 Lys Gly Ser Val Asn 250 Val Val Thr His Gly 255 Lys  
 Gly Leu Val Thr 260 Thr Leu His Glu Gly 265 Asp Asp Phe Gly Gln 270 Leu Ala  
 Leu Val Asn 275 Asp Ala Pro Arg Ala 280 Ala Thr Ile Ile Leu Arg Glu Tyr  
 Asn Cys 290 His Phe Leu Arg Val 295 Asp Lys Gln Asp Phe Asn Arg Ile Ile  
 Lys 305 Asp Val Glu Ala Lys 310 Thr Met Arg Leu Glu Glu His Gly Lys Val 320  
 Val Leu Val Leu Glu 325 Arg Ala Ser Gln Gly 330 Ala Gly Pro Ser Arg Pro 335  
 Pro Thr Pro Gly 340 Arg Asn Arg Tyr Thr 345 Val Met Ser Gly Thr Pro Asp 350  
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 His Val Glu Pro Ala 405 Gly Gly Ser Glu Gln 410 Glu Arg Ser Thr Tyr Val 415  
 Cys Asn Lys Arg 420 Gln Gln Ile Leu Arg 425 Leu Val Ser Gln Trp Val Ala 430  
 Leu Tyr Gly 435 Ser Met Leu His Thr 440 Asp Pro Val Ala Thr Ser Phe Leu 445  
 Gln Lys 450 Leu Ser Asp Leu Val 455 Gly Arg Asp Thr Arg 460 Leu Ser Asn Leu

- 37 -

Leu Arg Glu Gln Trp Pro Glu Arg Arg Arg Cys His Arg Leu Glu Asn  
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 485 490 495  
 Phe Leu Ser Ser Ala Pro Pro Gly Leu Gln Ala Pro Pro Ser Pro Pro  
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 Asp Pro Glu Gly Leu Cys Gly Arg Gly Lys Leu Ser Ser His Arg His  
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 Thr Pro Leu Pro Glu Gln Glu Gly Pro Thr Thr Gly Thr Val Gly Thr 35 40 45  
 ttt gaa ctg atg agc tcg aaa gac ttg gcg tac cag atg aca acg tat 191  
 Phe Glu Leu Met Ser Ser Lys Asp Leu Ala Tyr Gln Met Thr Thr Tyr 50 55 60  
 gac tgg gaa ctc ttc aac tgt gtg ctc gag ctg gag cta atc tac cac 239  
 Asp Trp Glu Leu Phe Asn Cys Val Leu Glu Leu Glu Leu Ile Tyr His 65 70 75  
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 Ile Lys Ile Ala Ala His Cys Lys Glu Tyr Lys Asn Leu Asn Ser Phe 130 135 140  
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- 38 -

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Pro Arg Pro  
290

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&lt;210&gt; 16

&lt;211&gt; 291

&lt;212&gt; PRT

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&lt;400&gt; 16

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- 41 -

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- 42 -

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- 43 -

&lt;210&gt; 18

&lt;211&gt; 849

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&lt;400&gt; 18

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- 44 -

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- 45 -

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Pro



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<b>(51) International Patent Classification <sup>7</sup> :</b> C12N 15/12, C07K 14/82, 16/32, 14/47, C12Q 1/68, C12N 5/10, A01K 67/027, G01N 33/53, 33/574	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 00/24768</b>  <b>(43) International Publication Date:</b> 4 May 2000 (04.05.00)
<b>(21) International Application Number:</b> PCT/US99/24826 <b>(22) International Filing Date:</b> 22 October 1999 (22.10.99)  <b>(30) Priority Data:</b> 60/105,507 23 October 1998 (23.10.98) US 60/108,685 16 November 1998 (16.11.98) US  <b>(71) Applicant (for all designated States except US):</b> MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02138 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> KAWASAKI, Hiroaki [JP/JP]; 3-20-2, Aoba, Higashi-ku, Fukuoka 813-0025, (JP). GRAYBIEL, Ann [US/US]; Boyce Farm Road, Lincoln, MA 01773 (US). HOUSMAN, David [US/US]; 64 Homer Street, Newton, MA 02158 (US).  <b>(74) Agent:</b> CAMACHO, Jennifer, A.; Testa, Hurwitz & Thibault, LLP, High Street Tower, 125 High Street, Boston, MA 02110 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>  <b>(88) Date of publication of the international search report:</b> 9 November 2000 (09.11.00)
<b>(54) Title:</b> GENES INTEGRATING SIGNAL TRANSDUCTION PATHWAYS  <b>(57) Abstract</b>  <p>The present invention describes the identification, isolation, sequencing and characterization of two human CalDAG-GEF, and two human cAMP-GEF genes, which are associated with the Ras pathway. Also identified are CalDAG-GEF gene homologues in mice and cAMP-GEF gene homologues in rats. Nucleic acids and proteins comprising or derived from the CalDAG-GEFs and/or cAMP-GEFs are useful in screening and diagnosing certain Ras-associated cancers, in identifying and developing therapeutics for treatment of certain Ras-associated cancers, and in producing cell lines and transgenic animals useful as models of Ras-associated cancers.</p>		

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## INTERNATIONAL SEARCH REPORT

Internat Application No  
PCT/US /24826

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C07K14/82 C07K16/32 C07K14/47 C12Q1/68  
C12N5/10 A01K67/027 G01N33/53 G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K C12Q A01K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EBINU, J.O. ET AL.: "RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs." SCIENCE, (1998 MAY 15) 280 (5366) 1082-6, XP000882708 the whole document	1
A	--- GOTOH, T. ET AL.: "Identification of Rap1 as a target for the Crk SH3 domain-binding guanine nucleotide-releasing factor C3G." MOLECULAR AND CELLULAR BIOLOGY, (1995) 15 (12) 6746-53, XP000881340 the whole document --- -/-	1

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

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Date of the actual completion of the international search

5 April 2000

Date of mailing of the international search report

3 July 2000 (03.07.00)

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Nichogiannopoulou, A

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KAIBUCHI, K. ET AL.: "Molecular cloning of the cDNA for stimulatory GDP/GTP exchange protein fo smg p21s (ras p21-like small GTP-binding proteins) and characterization o stimulatory GDP/GTP exchange protein." MOLECULAR AND CELLULAR BIOLOGY, (1991) 11 (5) 2873-80, XP000881341 the whole document	1
X	KEDRA D ET AL: "THE GERMINAL CENTER KINASE GENE AND A NOVEL CDC25-LIKE GENE ARE LOCATED IN THE VICINITY OF THE PYGM GENE ON 11Q13" HUM. GENET., vol. 100, 1 October 1997 (1997-10-01), pages 611-619, XP002069545 page 613, last paragraph -page 615, paragraph FIRST; figure 2 & DATABASE EMBL [Online] AC Y12336, 19 June 1997 (1997-06-19) KEDRA D ET AL: "H. sapiens mRNA for F25B3.3 kinase like protein from C. elegans" Protein with 96.9% identity to SEQ ID No:2 and 100% identity to SEQ ID No:4 the whole document	1,3,5-8, 10, 38-54, 63-69
P,X	WO 98 53061 A (QUEENSLAND INST MED RES; HANCOCK JOHN (AU); SILINS GINTERS (AU)) 26 November 1998 (1998-11-26)  MCG7, a hu protein with 100% identity in 609 aa overlap with SEQ ID No:4 claim 5; figure 13B	1,3,5-8, 10, 38-54, 63-69
P,X	KAWASAKI H ET AL: "A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia" PROC. NATL. ACAD. SCI. USA, vol. 95, October 1998 (1998-10), pages 13278-13283, XP000882748 the whole document	1,3,5-8, 10, 38-54, 63-69

# INTERNATIONAL SEARCH REPORT

Intern. application No.  
PCT/US 99/24826

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 76-81, 86-109 and 127 -in as far as they concern in vivo methods- are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
  
1, 3-8, 10, 12-19, 28-32, 38-70, 72, 73-75, 76, 78-82, 84, 86, 88-98, 110-112, 116, 118, 119, 121-129 (all partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1, 3-8, 10, 12-19, 28-32, 38-70, 72, 73-75, 76, 78-82, 84, 86, 88-98, 110-112, 116, 118, 119, 121-129 (all partially)

Claims relating to CalDAG-GEFI proteins and nucleic acids encoding them, mutants, variants, species orthologs and homologues, functional domains and antigenic determinants thereof. Methods for identifying variants or homologues of such proteins. Recombinant expression vectors, host cells and animal models for cancer expressing such recombinant modified proteins. Methods for producing said proteins and pure preparations of such. Antibodies selectively binding to such proteins, methods and cell lines for producing them. Methods for identifying compounds that modulate the expression of such proteins, and for identifying compounds that can selectively bind to them. Diagnostic methods for detecting mutations and pharmaceutical preparations comprising pure protein, expression vectors encoding the protein or antisense sequences. Pharmaceutical preparations comprising antibodies or antigenic determinants and methods of treatment.

2. Claims: 1, 3-8, 10, 12-19, 28-32, 38-70, 72, 73-75, 76, 78-82, 84, 86, 88-98, 110-112, 116, 118, 119, 121-129 (all partially)

As in subject 1, the proteins being CalDAG-GEFII proteins.

3. Claims: 2, 4-7, 9, 11, 20-27, 33-69, 71-75, 77-81, 83, 85, 87, 99-109, 113-115, 117, 118, 120-126, 130 (all partially)

As in subject 1, the proteins being cAMP-GEFI proteins.

4. Claims: 2, 4-7, 9, 11, 20-27, 33-69, 71-75, 77-81, 83, 85, 87, 99-109, 113-115, 117, 118, 120-126, 130 (all partially)

As in subject 1, the proteins being cAMP-GEFII proteins.



# INTERNATIONAL SEARCH REPORT

Internati- [REDACTED] Application No

PCT/US 99/24826

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9853061 A	26-11-1998	AU 7512998 A	11-12-1998
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